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## RIT GTPASE SIGNALING MEDIATES OXIDATIVE STRESS RESISTANCE AND SURVIVAL OF ADULT NEWBORN NEURONS AFTER TRAUMATIC BRAIN INJURY

Weikang Cai

University of Kentucky, wcai3@uky.edu

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Weikang Cai, Student

Dr. Douglas A. Andres, Major Professor

Dr. Kevin D. Sarge, Director of Graduate Studies

RIT GTPASE SIGNALING MEDIATES OXIDATIVE STRESS RESISTANCE AND  
SURVIVAL OF ADULT NEWBORN NEURONS AFTER TRAUMATIC BRAIN  
INJURY

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DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Medicine at the University of Kentucky

By

Weikang Cai  
Lexington, Kentucky  
Director: Dr. Douglas A. Andres, Professor of Biochemistry  
Lexington, Kentucky  
2011

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## ABSTRACT OF DISSERTATION

### RIT GTPASE SIGNALING MEDIATES OXIDATIVE STRESS RESISTANCE AND SURVIVAL OF ADULT NEWBORN NEURONS AFTER TRAUMATIC BRAIN INJURY

The small GTPases function as molecular switches to control diverse signaling cascades. The mammalian Rit and Rin, along with *Drosophila* Ric, comprise an evolutionarily conserved subfamily of the Ras-related GTPases. Previous studies using cultured cell models suggested that Rit was involved in the control of cell proliferation, transformation, neuronal differentiation, morphogenesis, and cell survival, but the principal physiological function of Rit remained uncharacterized.

To address this outstanding question, we employed a genetic approach, engineering a Rit knockout mouse. Using this animal model, we demonstrate a central role of Rit in governing cell survival in a p38-dependent fashion. Primary mouse embryonic fibroblasts (MEFs) derived from Rit<sup>-/-</sup> mice display increased apoptosis and selective disruption of MAPK signaling following oxidative stress. These deficits include a reduction in ROS-mediated stimulation of a novel p38-MK2-HSP27 signaling cascade, which appears to act upstream of the mTORC2 complex to control Akt-dependent cell survival.

In the adult brain, proliferation of stem cells within the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), provide a lifelong supply of new neurons. Adult neurogenesis appears critical for learning and memory and is altered in animal models of brain injury and neurological diseases. Thus, a greater understanding of the regulation of adult neurogenesis will provide insight into its myriad physiological roles but also to the development of therapeutic strategies for the treatment of injury and the progression of brain diseases. Here we find that Rit plays a central role in governing the survival of hippocampal neurons in response to oxidative stress. Importantly, using a controlled cortical impact model of traumatic brain injury (TBI), we show that Rit acts to protect newborn immature neurons within the SGZ of the DG from apoptosis following TBI. Finally, studies indicate that Rit plays a significant role in directing IGF-1 signaling, a key neurotrophin known to promote neurogenesis and to protect neurons against apoptotic stress.

Together, these studies establish Rit as a critical regulator of a p38 MAPK-dependent signaling cascade that functions as an important survival mechanism for cells



in response to oxidative stress, including the survival of newborn hippocampal neurons in the traumatically injured brain.

KEYWORDS: Rit, small GTPase, oxidative stress, p38 MAPK, TBI

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Weikang Cai

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11/18/2011

RIT GTPASE SIGNALING MEDIATES OXIDATIVE STRESS RESISTANCE AND  
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INJURY

By

Weikang Cai

Douglas A. Andres, Ph.D.  
Director of Dissertation

Kevin D. Sarge, Ph.D.  
Director of Graduate Studies

11/18/2011

*To my parents, Zhenguo Cai and Rongqin Tang, and my wife, Louise Jiang*



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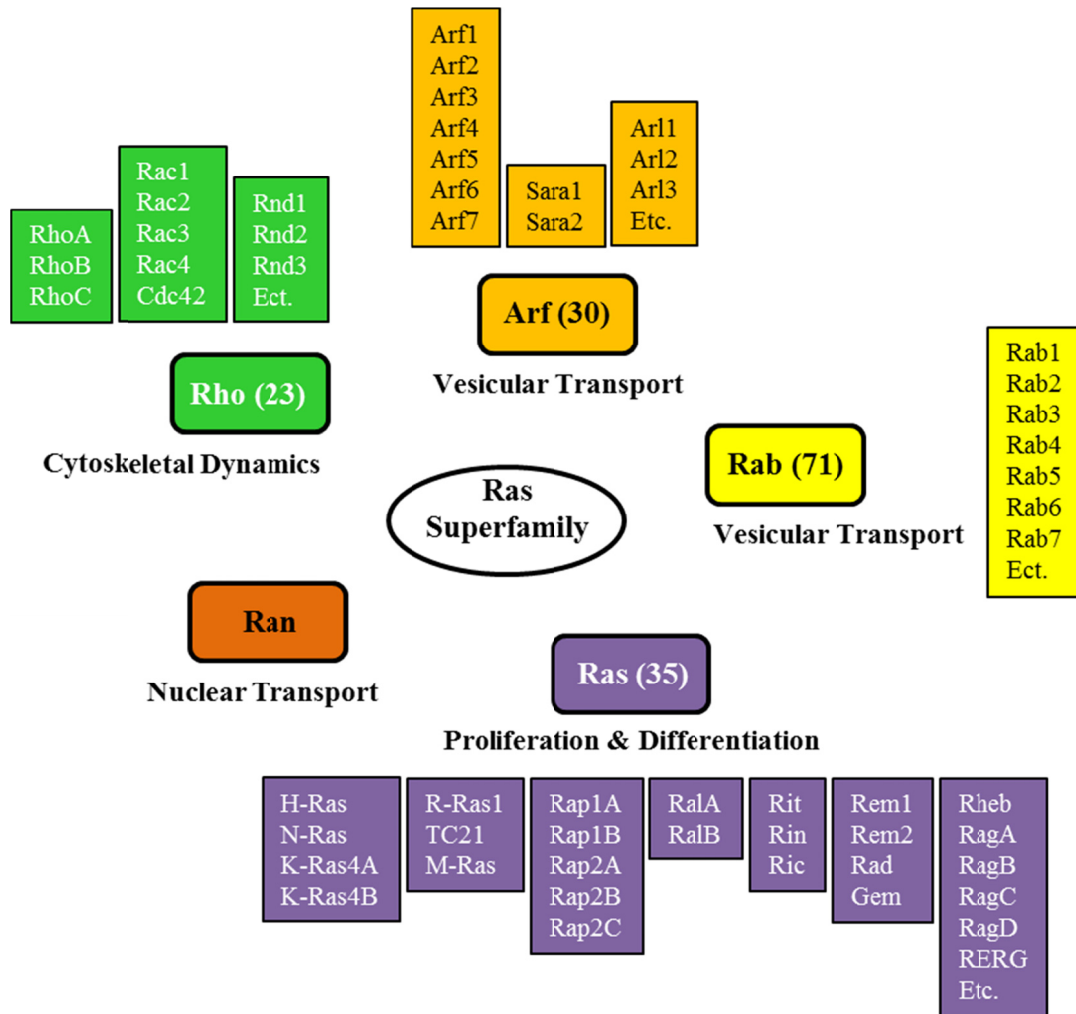
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## CHAPTER ONE

### Introduction

#### Ras Superfamily

The Ras superfamily is comprised of a group of low molecular weight guanine nucleotide binding proteins, also known as small GTPases, which function as molecular switches, responding to a wide variety of extra- and intra-cellular stimuli to control the activity of diverse signaling cascades [1]. To date, over 150 different small GTPases have been identified and are classified into five distinct subfamilies: Ras (Rat sarcoma), Rho (Ras homolog gene family), Rab (Ras-related GTP-binding protein), Arf (ADP-ribosylation factor) and Ran (Ras-related nuclear protein), based upon both sequence homology and the regulation of common cellular functions (Fig. 1.1) [2]. Generally, Ras subfamily proteins are critical in cell growth and proliferation, differentiation and survival [3, 4]. Rho GTPases are important in modulating cytoskeletal dynamics [5]. Rab and Arf proteins are key regulators in vesicular trafficking [6, 7], while Ran proteins play pivotal roles in nucleo-cytoplasmic transport [8]. While the function of a large portion of small GTPases are known, the physiological functions of many other members remain to be identified. This is particularly true within Ras subfamily, where a number of “orphan” GTPases have not been extensively studied. The identification of the physiological function of the Rit GTPase is the goal of this thesis. Despite the distinct cellular functions, all the small GTPases share a highly conserved core structure which is responsible for guanine nucleotide binding and hydrolysis [9]. This conserved core ensures that the majority of the small GTPases function as molecular switches that can be turned on and off depending on the state of bound nucleotide (GTP/GDP) in response to extra- and intra-cellular stimuli. These conserved functional properties lead to the positioning of Ras superfamily proteins as the critical control step in numerous signal transduction cascades.



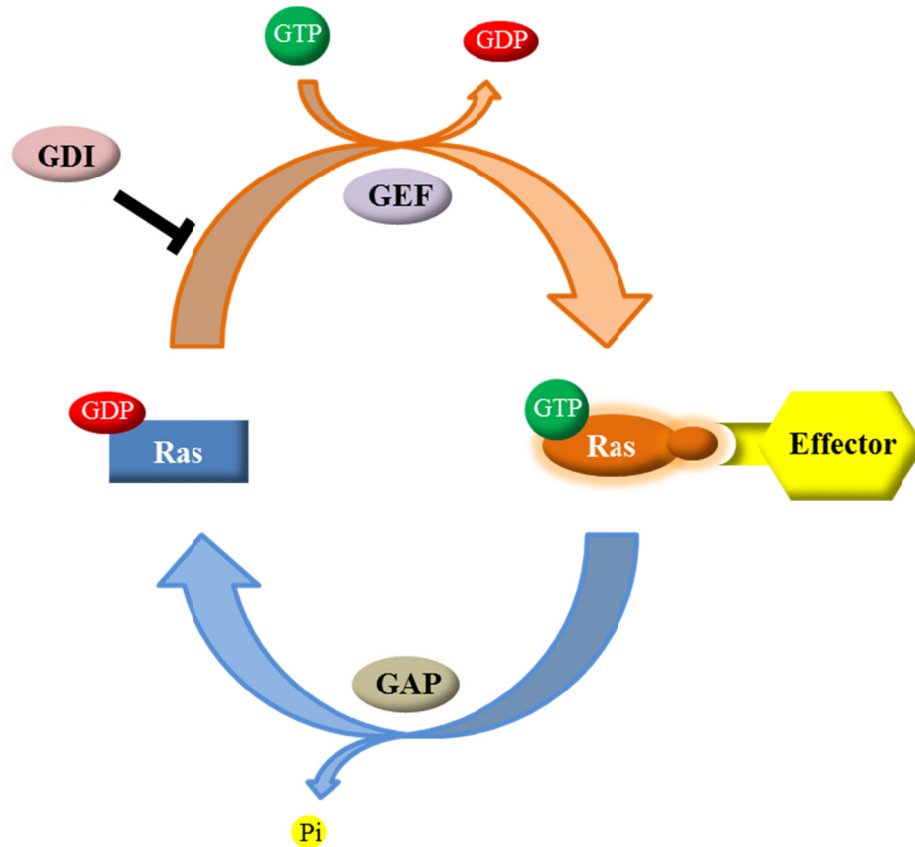
**Figure 1.1 The Ras Superfamily of Small GTPases.** Members of the Ras superfamily GTPases (>150) are classified into five broad families, based on both sequence homology and the regulation of common cellular functions. They are Ras, Rho, Rab, Arf and Ran families.

## The GTPase Cycle

As binary molecular switches, Ras-like small GTPases can cycle between an inactive, GDP-bound conformation and an active, GTP-bound structural state (Fig. 1.2). GTP binding results in a conformational change in the core structure which exposes an effector binding loop (the G2 or effector loop), allowing the binding of downstream effector molecules to promote the activation of downstream signaling pathways. By hydrolyzing bound GTP to GDP, due to the intrinsic GTPase activity of Ras proteins, the inactive conformation of the effector loop is restored, resulting in the loss of effector binding and subsequently the inactivation of downstream signaling cascades [9, 10].

The activation of small GTPases requires the exchange of bound GDP for GTP. Since its binding affinity for guanine nucleotide is high (with  $K_d$  value at  $10^{-11}$  M range) [10], uncatalyzed nucleotide exchange, which would result in spontaneous activation of Ras proteins, is not expected to occur under normal physiological conditions. Meanwhile, the low intrinsic GTPase activity suggests that Ras-like superfamily proteins undergo only slow inactivation[10]. These functional properties of the biotimer switch indicate that Ras-like small GTPases cannot act alone to regulate rapid and efficient signaling cascades. Indeed, additional regulatory proteins are necessary for both temporal and spatial regulation of Ras-like small GTPase activity. A family of guanine nucleotide exchange factors (GEFs) binds to the inactive GDP-bound conformational state of select GTPase proteins and accelerates their release of bound nucleotide [11, 12]. Since the intracellular GTP concentration is approximately 10-fold higher than GDP, nucleotide-free GTPases rapidly bind free GTP molecules upon GEF disassociation, resulting in GTPase activation. On the other hand, GTPase-activating proteins (GAPs) are able to stimulate the usually low GTPase activity of Ras proteins by up to 1000 fold, leading to a rapid GTP hydrolysis, and a return to the GDP-bound inactive state [13]. Therefore, the combined actions of GEFs and GAPs allow Ras-like small GTPases to be subjected to rapid and appropriate regulation in response to upstream stimuli. Guanine nucleotide dissociation inhibitors (GDIs) have been identified for the Rho and Rab subfamilies [14]. Like GEFs, GDIs preferentially bind to GDP-bound inactive GTPase. However, instead of stimulating the nucleotide exchange and activating GTPases, GDIs are known to prevent GEF binding and thus retain the GTPases inactive. In addition, GDIs are also

shown to block the membrane targeting of Rho and Rab subfamily proteins [14]. Together, the GTPase cycle and these diverse regulatory proteins ensure the simple but tight regulation of GTPases, which makes GTPases critical targets in controlling complex signaling cascades.



**Figure 1.2 The Small GTPase Cycle.** Small GTPase cycles between a GDP-bound inactive state and a GTP-bound active state. GTP-binding triggers a conformational change allowing the binding of downstream effectors. Guanine nucleotide exchange factor(s) (GEFs) stimulates the release of GDP, which is rapidly replaced by GTP. GTPase-activating protein(s) (GAPs), on the other hand, accelerate the intrinsic GTPase activity of small G-proteins, increasing the rate of GTP hydrolysis to GDP. Guanine nucleotide dissociation inhibitor (GDI) prevents GEF binding and subsequent GTPase activation, particularly for Rho and Rab family members.

## **Ras Structure and Biochemical Characteristics**

All Ras superfamily proteins have a high degree of amino acid conservation within the core domain responsible for nucleotide binding and hydrolysis, including five conserved structural regions (G1-5 domains) [2, 10, 15]. The G1 domain, also known as the Walker A motif or P loop, is responsible for interacting with the  $\alpha$ - and  $\beta$ -phosphate groups of purine nucleotides [16]. The G3 domain is responsible for binding the  $\gamma$ -phosphate group of GTP as well as the nucleotide-associated  $Mg^{2+}$  ion [10]. G4 and G5 domains can specifically recognize and bind the guanine ring, which confers specificity for GTP over ATP [2]. Lastly, the G2 domain, also known as the “effector domain”, serves as the primary interaction site with downstream effector proteins following G-protein activation, allowing individual G-proteins to recognize distinct effector proteins [2]. G2 and G3 are also called the switch I and II domains, both of which undergo dramatic conformation changes depending on whether the  $\gamma$ -phosphate of GTP is present, in other words, whether GTP or GDP is bound. When GTP is bound, these two loops are exposed to the surface of the GTPase core and capable of recruiting effectors and activating downstream signaling cascades. On the other hand, GTP hydrolysis triggers conformational changes to both switch I and II, which bury these loops to end effector binding and terminate downstream signaling [9].

With insight gained from the crystal structures of Ras proteins complexed with non-hydrolyzable GTP analogs as well as the GAP catalytic domain, the mechanism of GTP hydrolysis by small GTPases is well characterized. Gln<sup>61</sup> residue in the switch II region in Ras, for example, acts as a general base to facilitate the nucleophilic attack of the  $\gamma$ -phosphate of GTP by a water molecule in the active site [17]. Since the switch II region is flexible, this critical Gln<sup>61</sup> residue cannot always be positioned at the perfect orientation to catalyze GTP hydrolysis, which explains the low intrinsic GTPase activity of Ras proteins. Binding to GAP proteins, however, dramatically facilitates the GTP hydrolysis. Upon binding, GAP inserts an additional catalytic Arginine residue into the Ras active site, which stabilizes the transition state to promote GTP hydrolysis. During hydrolysis, another residue Gly<sup>12</sup> within the G1 domain is also involved in stabilization of the transition state [18, 19]. Therefore, mutation of either Gln<sup>61</sup> or Gly<sup>12</sup> is sufficient to disrupt GTP hydrolysis and results in a GAP-insensitive Ras mutant, which will always



be locked in the active state. Indeed, both Ras<sup>Q61L</sup> and Ras<sup>G12V</sup> mutants are considered to be “constitutively active” and have been used extensively to study Ras-mediated signaling cascades and biological processes. On the other hand, mutation of the Ser<sup>17</sup> residue in the G1 domain of Ras leads to a 40-fold decrease in GTP binding affinity without significantly affecting GDP binding, thereby locking the Ras protein into the GDP-bound inactive state [16, 20]. Overexpressing this “dominant negative” Ras<sup>S17N</sup> mutant in the cells will compete with endogenous Ras proteins for upstream regulatory proteins (particularly Ras GEFs), thus blocking the signals to stimulate GTPase activity. Dominant negative mutant of GTPases have also proven to be invaluable reagent in deciphering Ras family signaling pathways.

### **Cellular Distribution and Trafficking**

For the majority of the Ras-related small GTPases, association with the cellular membrane is essential for their biological activity [10]. Usually, two distinct membrane targeting signals are required for the efficient membrane localization of small GTPases, with a few exceptions in which only one signal is present [21]. For the majority of the G-proteins, membrane targeting requires post-translational lipidation, including modification with farnesyl or geranylgeranyl isoprenoids (prenylation), palmitate (palmitoylation) and/or myristate (myristoylation) [2, 22]. The most common prenylation signal is a conserved tetrapeptide motif, C-A-A-X, which resides at the extreme C-terminus [1]. Here, “C” is Cysteine, “A” is aliphatic amino acid and “X” is variable. The CAAX box can direct either farnesylation if the “X” is anything but leucine or geranylation if “X” is leucine. Following prenylation, the AAX tripeptide is removed and the now C-terminal prenylated cysteine residue is carboxymethylated. Prenylation is only the initial step in the attachment of these Ras-like small GTPases to the cytoplasmic leaflets of a variety of cellular organelles. Specific membrane localization often requires additional targeting signals, provided either by a cluster of basic amino acids or the palmitoylation of internal cysteine residues [23]. For instance, adjacent to the CAAX prenylation motif, H-Ras and N-Ras have a cysteine residue that is subject to palmitoylation, leading to strong membrane localization. In contrast, K-Ras4A has a polybasic motif in the C-terminus, in which positively charged lysine and/or arginine

residues are recruited to negatively charged phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>) and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>) lipids at the plasma membrane [24]. Furthermore, some other small GTPases, such as Arf family members, lack a C-terminal prenylation motif. Instead, they are myristoylated on an N-terminal glycine residue, which directs plasma membrane association [22]. Finally, as mentioned above, there are a few small GTPases in which the presence of a large polybasic domain directs membrane localization, including Rad/Gem/Kir (RGK) subfamily members and Rit subfamily GTPases [21].

### **Ras Subfamily**

Within Ras subfamily, there are at least 35 members, all of which contain related G2 effector domains [2]. These include the Ras proteins (H-Ras, K-Ras4A/B and N-Ras), Rap proteins (Rap1A/B, Rap2A/B/C), R-Ras-like proteins (R-Ras1, R-Ras2/TC-21, and R-Ras3/M-Ras), Ral proteins (RalA/B), RGK proteins (Rem, Rem2, Rad, and Gem), Rit proteins (Rit, Rin and *Drosophila* Ric), and Rheb among others (Fig. 1.1). Despite the high sequence homology and highly related G2 effector domains, each group has been shown to possess overlapping but distinct physiological functions [2]. In this session, I will review the most common properties as well as functions of Ras subfamily proteins taking the most famous member Ras as an example.

### **Ras Biological Activities**

Ras genes were first discovered as the oncogenes present within the Harvey (H-Ras) and Kirsten (K-Ras) murine sarcoma viruses [10]. Activated Ras mutant proteins (H-Ras, K-Ras, as well as N-Ras) were subsequently identified in approximately 30% of all human tumors, suggesting a critical regulatory role for Ras proteins in tumor development/cell transformation [2]. This is further supported by the fact that overexpression of constitutively active Ras mutants can transform NIH-3T3 fibroblasts [15]. However, active Ras mutants alone are not sufficient to transform primary cells [25]. The transformation of primary cell can only occur when active Ras mutations coincide with the activation of “immortalizing” oncogenes such as myc or SV40 large T antigen [26], indicating that active Ras alone is not sufficient to promote transformation,

but instead is one critical aspect of a series of mutational events that under tumor formation.

With the more intensive investigation focused on understanding their role in tumorigenesis, Ras proteins have now been shown to play diverse cellular functions beyond cancer cell growth. These include a role for Ras GTPases in cell proliferation, both positive (cell cycle progression) and negative (cell cycle arrest). The first evidence of Ras involvement in proliferation came from studies in which microinjection of neutralizing monoclonal antibody against Ras into NIH-3T3 was found to inhibit serum or growth factor-induced mitogenesis [27, 28]. This is further supported by the finding that mitogens stimulate cell proliferation by regulating the level of cell cycle proteins such as cyclin D1 and p21<sup>CIP1</sup> through Ras-mediated transcriptional regulation [29, 30]. The role for Ras signaling in cell cycle regulation is complicated by studies showing that expression of active Ras is able to cause permanent cell cycle arrest (cellular senescence) in cultured primary fibroblasts [31], although whether Ras-induced senescence occurs *in vivo* remains under debate.

Besides its role in regulating cell proliferation, Ras proteins are also known to induce differentiation in certain cell types, such as induction of neurite outgrowth in the rat pheochromocytoma PC12 cell line [32]. In the presence of Nerve Growth Factor (NGF), PC12 cells exit the cell cycle and differentiate into a sympathetic neuron-like phenotype [33]. Overexpressing constitutively active Ras induces neurite outgrowth similar to that seen following NGF stimulation [32]. Moreover, expression of dominant negative Ras blocks NGF-induced neurite outgrowth [34], further demonstrating the pivotal role of Ras signaling in neuronal differentiation. In addition, Ras has also been implicated in other biological processes including cell survival and neuronal plasticity and long-term memory [25, 35, 36].

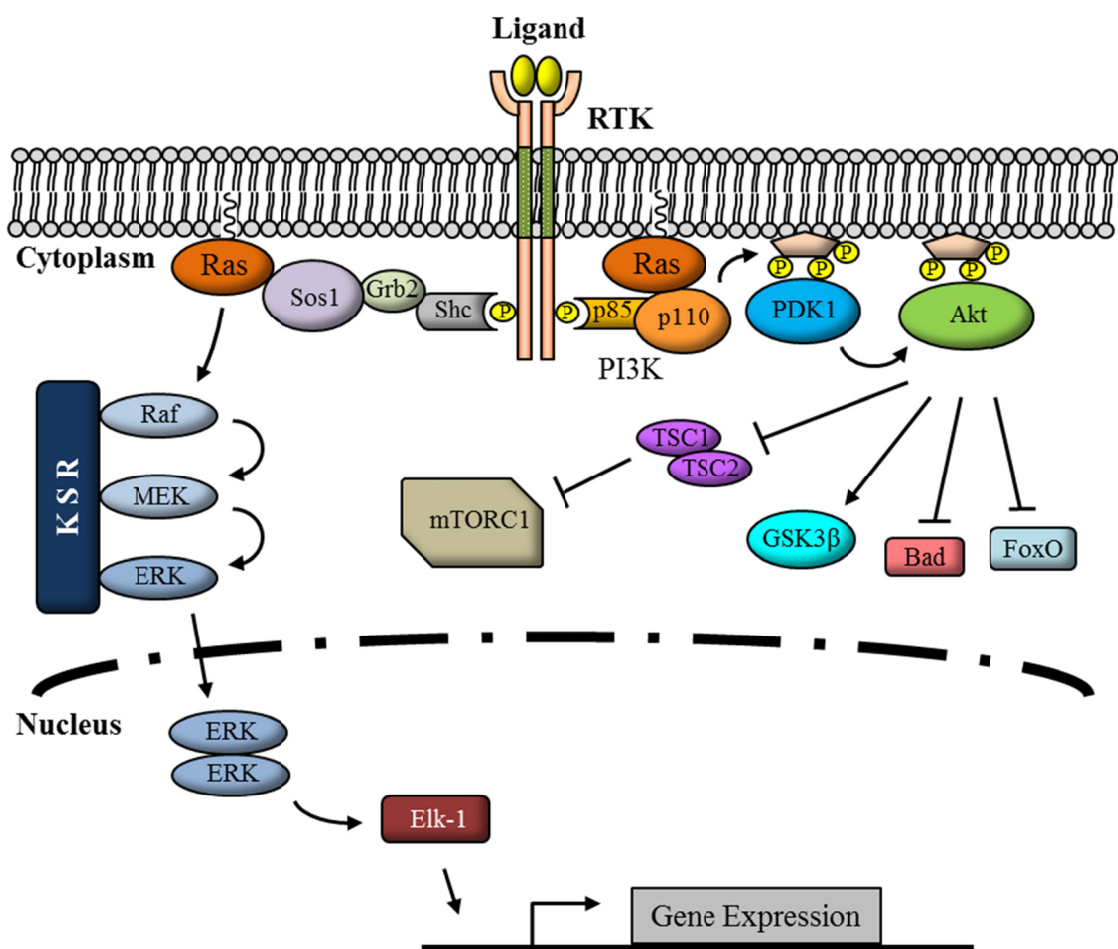
### **The Upstream Regulators of Ras**

Ras proteins are activated by a wide spectrum of extracellular stimuli, including ligand activated cell surface receptors (such as seven transmembrane-spanning G-protein coupled receptors, receptor tyrosine kinases (RTKs), and cytokine receptors), and intracellular stresses [37]. Among these signaling cascades, activation of Ras following

RTK stimulation is best characterized (Fig. 1.3) [37]. Upon ligand binding, receptor tyrosine kinases dimerize, which leads to autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptor. These phosphorylated tyrosine residues in turn serve as docking sites for SH2 (Src homology 2) domain-containing adaptor proteins, such as Shc/Grb2. Grb2 then interacts with the proline-rich region of Ras GEFs, such as Sos1 (son of sevenless 1), through an SH3 (Src homology 3) domain. As a result, Ras GEFs are recruited to the plasma membrane, where they come in contact with Ras proteins and stimulate the exchange of GTP for GDP, leading to Ras activation [37].

As discussed previously, GEFs are direct activators of Ras proteins, and therefore every cellular stimulus that results in Ras activation requires a GEF regulatory protein. Therefore, the identification of Ras GEFs is of great importance to understanding Ras signaling. The yeast Cdc25 (cell division cycle 25) protein was the first Ras GEF to be identified [38]. Since then, a large number of mammalian GEFs have been identified for the Ras subfamily, including Sos, RasGRF2, Ras GRP and C3G [12, 39, 40], collectively forming the Cdc25 family of RasGEFs. Different GEFs display distinct substrate specificity. For instance, Sos preferentially stimulates Ras and Rac activation [41], while C3G is known to preferentially activate Rap proteins [42].

In order to shut down Ras signaling rapidly and appropriately, RasGAP proteins are required to stimulate rapid hydrolysis of bound GTP. At present, six different RasGAPs have been discovered in mammalian cells: p120GAP [43], the neurofibromatosis type 1 protein (NF1) [44], GAP1m, GAP1IP4BP [45], SynGAP [46], and IQGAP1 [47]. All of these proteins bind preferentially to GTP-bound Ras and are able to enhance intrinsic GTPase activity to terminate Ras signaling. Indeed, loss of function mutations of GAPs can result in aberrant, and sustained Ras signaling, leading to tumorigenesis such as neurofibromatosis, or a variety of developmental disease including Noonan syndrome among others [25, 48].



**Figure 1.3 RTK-Mediated Ras-ERK MAPK Cascade and PI3K-PDK1-Akt Pathway.** Ligand binding induces the dimerization of RTKs, which triggers autophosphorylation of tyrosine residues located on the cytoplasmic portion of the receptor. Once phosphorylated, adapter proteins such as Shc/Grb2 binds in a sequence dependent fashion to specific phosphorylated tyrosine residues via SH2 domains and recruit additional proteins to the activated receptor complex. Among these proteins are GEFs, such as Sos1, which once in proximity to the plasma membrane activates Ras small GTPases. The GTP-loaded Ras, in turn, activates the Raf/MEK/ERK signaling cascade scaffolded by KSR. Phosphorylated ERK proteins dimerize and translocate into the nucleus, where they activate transcription factors such as Elk-1 to control gene expression. PI3K can also be recruited to specific phospho-tyrosine residues on the activated RTK through the SH2 domain located in the p85 regulatory subunit. Meanwhile, Ras small GTPases can also directly interact with p110 catalytic subunit in a GTP-dependent manner. Both mechanisms have been proven crucial for PI3K activation. Once activated, PI3K produces  $PI(3,4,5)P_3$ , which is responsible for the membrane recruitment of PDK1 and Akt via PH domains. Anchored onto the membrane, PDK1 phosphorylates and activates Akt, which in turn phosphorylates multiple downstream targets and control diverse cellular functions, including cell survival.

### **The Effector Pathways of Ras**

Activated Ras proteins interact with a diverse array of downstream effector proteins, allowing regulation of a wide range of signaling cascades [49]. At present, a number of Ras effector proteins have been identified by a variety of approaches, including genetic screens in *Drosophila* and *C. elegans*, biochemical binding assays and yeast two-hybrid screening. These effector proteins include Raf kinases, PI3K, RalGDS family proteins, Ras-association containing protein family (RASSF), phospholipase C- $\epsilon$  (PLC $\epsilon$ ), Ras interaction protein-1 (RIN1), ALL (acute lymphoblastic leukaemia)-1 fused gene on chromosome 6 (AF-6), etc [50, 51]. Through the regulation of this diverse collection of effector proteins, Ras proteins control a variety of cellular activities, including proliferation, differentiation, cell survival and death [25].

### **The Ras Effector Domain**

The effector domain (G2 domain) of the Ras GTPases is responsible for the recognition and interaction with the downstream effector proteins [2]. Upon GTP binding, the G2 effector domain undergoes a conformational change, allowing effector protein binding. As discussed above, a variety of studies have revealed that individual Ras protein can act on multiple effector proteins. For instance, Ras signals through the activation of Raf1, PI3K, RalGDS, RIN1, AF6, etc. when GTP-bound [49]. This suggests that different residues within the effector domain are likely responsible for directing the association with different effector proteins. Indeed, analysis of Ras effector domain mutants has revealed point mutations that preferentially bind and activate one single downstream effector pathway. Using Ras as an example of this approach, Thr<sup>35</sup> and Gln<sup>38</sup> residues have been shown to be required for PI3K and RalGDS binding, Glu<sup>37</sup> is important for Raf1 and PI3K binding, while Tyr<sup>40</sup> is critical for Raf1 and RalGDS binding [52]. These effector domain mutants of Ras proteins have been proved useful for determining the relative contribution of individual effector pathways to Ras function. For example, Rodriguez-viciana, *et al.* identified the critical role of PI3K in actin cytoskeleton rearrangement and cell transformation using this approach [52].

### **MEK/ERK MAPK Cascade**

The mitogen activated protein kinase (MAPK) cascades are well defined downstream effector pathways for a variety of Ras subfamily GTPases [49]. MAPK cascades are composed of three levels of protein kinases: MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAP kinase (MAPK) [53]. The activation of MAPK cascades involves the sequential phosphorylation of all three protein kinases. The first mammalian effector of Ras identified was the serine/threonine protein kinase Raf, which is a MAPKKK [54]. There are three closely related Raf proteins: A-Raf, B-Raf, and C-Raf (Raf-1), all of which display high binding affinity to GTP-bound Ras with an intact effector domain. The binding of Raf to plasma membrane anchored GTP-bound Ras relocates Raf from the cytoplasm. The full activation of Raf is contributed by a complex mechanism involving Ras binding in combination with association with scaffolding protein like kinase suppressor of Ras (KSR) and the action of a variety of additional cellular kinases [55]. Active Raf kinases then phosphorylate their direct substrates MEK1/2 (MAPKK). The dual specificity MEK1/2 kinases, in turn, phosphorylate the tandem threonine and tyrosine residues of ERK1/2 (MAPK) [53]. Upon phosphorylation, ERK1/2 homodimerizes and translocates into the nucleus, where they directly or indirectly phosphorylate a variety of transcriptional factors, such as Elk-1 and c-Jun, to activate gene expression, especially the expression of genes involved in cell cycle progression/cell proliferation (Fig. 1.3) [56].

To enhance the efficiency and specificity of MAPKKK/MAPKK/MAPK cascade signaling, all three kinases are often found pre-assembled on a “scaffolding” protein. For instance, KRS is able to bind to Raf, MEK1/2 and ERK1/2, allowing this multidomain protein to exert substantial control over ERK signaling, influencing the signal intensity, time course, and importantly, the nature of the cellular response (Figure 1.3) [55]. Therefore, scaffolding complexes allow modulation of MAPK signaling and help to regulate fundamental cellular processes.

Besides ERK1/2, there are two other major MAPKs: p38 MAPK and JNK (c-Jun N-terminal kinase) [53]. Similar to ERK, both p38 and JNK MAPK pathways have equivalent three level kinase cascades, and both kinase cascades are downstream effector pathways for activated Ras proteins [53]. However, the cellular functions of these three



MAPK pathways are overlapping, but not identical. While ERK signaling is critical in mitogen signaling, p38 and JNK pathways are thought to play a major role in stress response and other pathways through the regulation of a distinct array of transcription factors [57, 58].

### **p38 MAPK Pathway**

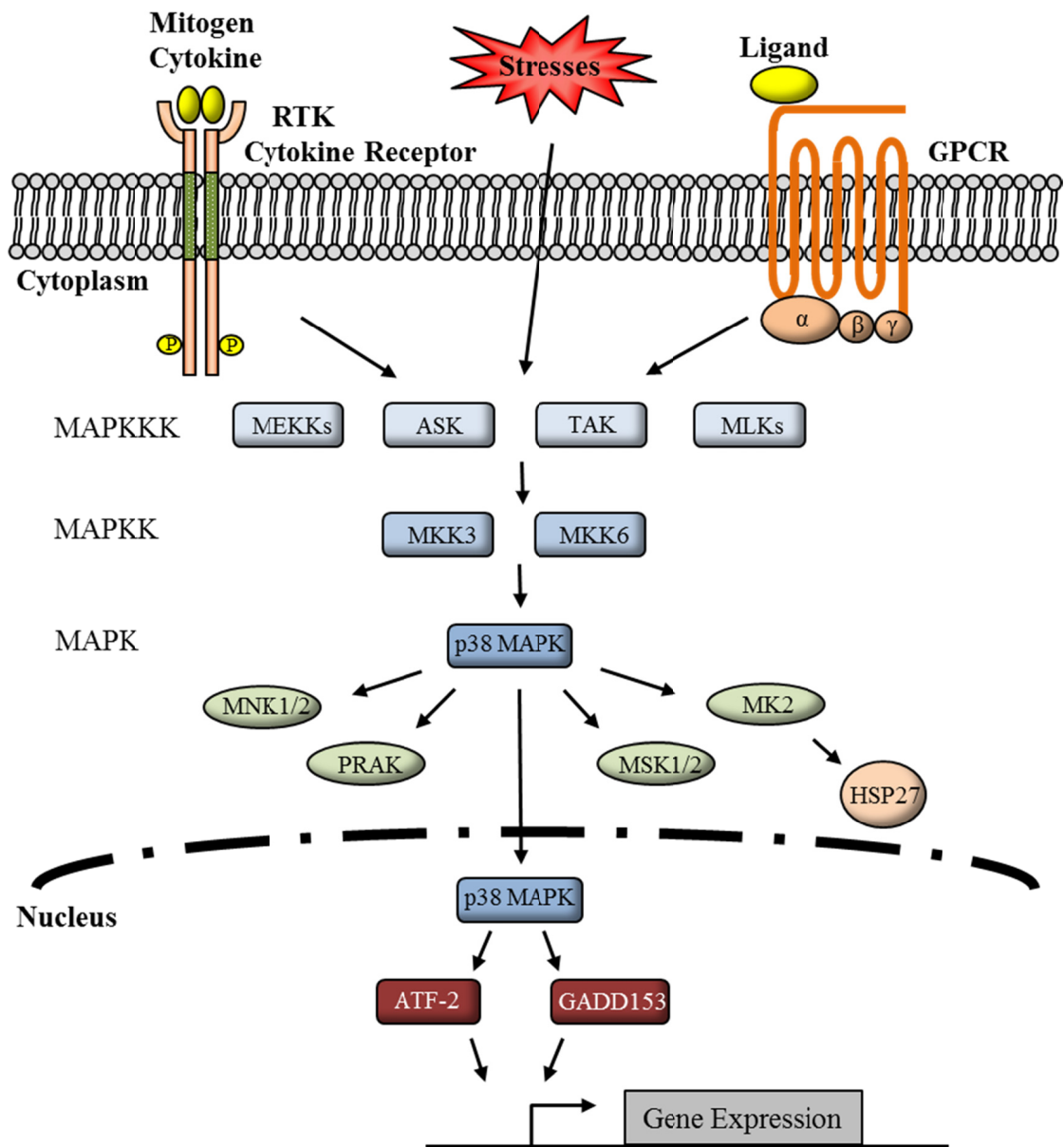
p38 MAPKs, along with JNK, are also called stress-activated protein kinases (SAPKs) since they respond to various environmental and cellular stresses, such as osmotic stress, UV irradiation, hypoxia, oxidative stress and pro-inflammatory cytokines, although they are also activated by a number of growth factors [57]. Initially, p38 MAPKs were extensively studied in cytokine-mediated signaling and considered important in inflammatory responses. However, later studies revealed that p38 MAPKs play critical roles beyond the stress response, including development, differentiation, proliferation and survival/apoptosis [59, 60]. p38 MAPKs are comprised of four isoforms in mammalian systems: p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$  and p38 $\delta$ . However, these kinases share only about 60% sequence identity, suggesting that each may control diverse cellular functions [58]. This notion is supported by the distinct expression patterns of the isoforms. p38 $\alpha$ / $\beta$  are ubiquitously expressed, whereas p38 $\gamma$ / $\delta$  expression is more tissue restricted. p38 $\gamma$  is enriched in muscle and p38 $\delta$  is highly expressed in lung and kidney [59]. Since most of available reagents are specific for the p38 $\alpha$  and  $\beta$  isoforms, including selective pharmacological inhibitors (for example SB203580), both p38 $\alpha$  and p38 $\beta$  are much better studied than the remainder of the family [61].

Similar to the ERK cascade, the activation of p38 MAPKs involves a three-level hierarchical cascade (MAPKKK/MAPKK/MAPK) [53]. p38 MAPKs can be activated by dual specificity MAPKKs, MKK3/6, which phosphorylate a TGY module in the activation loop [57]. Unlike ERK, the p38 cascade is regulated by a large number of MAPKKKs, including MEKKs, apoptosis signal-regulating kinase 1 (ASK1), transforming growth factor  $\beta$ -activated kinase 1 (TAK1), and mixed lineage kinases (MLKs), all of which have been shown to activate MKK3/6 and ultimately regulate p38 activity (Fig. 1.4) [57]. This reverse pyramidal structure for the p38 cascade indicates that a large number of different upstream stimuli (including both cellular stresses and

extracellular ligands) utilize specific MAPKKKs to feed into this kinase signaling pathway, which puts p38 at a key convergent regulatory point for a wide range of intracellular signaling networks.

To facilitate signal transduction specificity, scaffolding proteins are also involved in p38 signaling [62]. For instance, the OSM scaffold binds to MEKK3, MKK3 and p38 MAPK. In response to osmotic stress, OSM is recruited to the plasma membrane and binds to the activated Rac GTPase [62]. Following assembly of the scaffolded complex, p38 MAPK is rapidly and spatially activated. This complex is conserved from yeast to mammals, suggesting its evolutionary importance [62]. In addition, some scaffolding proteins for JNK, such as JNK-interacting proteins (JIPs), also appear to function in the regulation of p38 MAPK signaling. JIP2 scaffolds a complex that contains the Rac GEFs Tiam and RasGRF1, MLK3, and MKK3 to specifically activate p38 MAPK [63].

As a serine/threonine protein kinase, p38 MAPK acts by phosphorylating and subsequently regulating the activity of its direct substrates. These include transcription factors, a second tier of kinases, and phosphatases [64]. For example, p38 phosphorylates the ATF-2 transcription factor, which can form homodimers or heterodimers with c-Jun, therefore, regulate downstream gene targets [65]. p38 MAPK is also known to phosphorylate Sap-1a and growth arrest and DNA damage transcription factor 153 (GADD153) [66, 67]. In addition to directly regulating gene transcription, p38 can also control signaling pathways by phosphorylating kinases including mitogen activated-protein kinase activated protein kinases (MAPKAPKs, MK2/3) [68], mitogen- and stress-activated protein kinases (MSK1/2) [69], mitogen-activated protein kinase-interacting kinases (MNK1/2) [70] and PRAK (also called MK5) [71]. Among these, MK2 is one of the best characterized substrate kinases. p38 and MK2 constitutively form a heterodimer and shuttle between the cytoplasm and nucleus, where they are able to phosphorylate a number of downstream targets upon stimulation [72]. These substrates include the small heat shock protein HSP27, which may contribute to the regulation of cytoskeleton dynamics, protein folding, and cell survival [73, 74].



**Figure 1.4 p38 MAPK Signaling Pathway.** A variety of extracellular stimuli including mitogens, cytokines as well as environmental stresses are known to activate p38 MAPK signaling. p38 is primarily regulated by the MAPKKs, MKK3 and MKK6, whose kinase activities are controlled by a diverse array of MAPKKKs. Upon activation, p38 MAPK directly phosphorylates downstream targets such as MNK1/2, PRAK, MK2/3 and MSK1/2. p38 can also translocate into the nucleus to phosphorylate and regulate transcription factors, including ATF-2 and GADD153.

### **PI3K Pathway**

Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase which catalyzes the phosphorylation of phosphatidylinositol 3,4-bisphosphate (PI(3,4)P<sub>2</sub>) at the 3' position of the inositol ring to produce phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>), which is a well-known intracellular second messenger that binds to a group of proteins through their conserved pleckstrin homology (PH) domains [75]. PI3K is composed of a p110 catalytic subunit and a p85 regulatory subunit (Fig. 1.3). The p110 catalytic subunit can directly interact with Ras GTPase in a GTP- and effector domain-dependent manner, making PI3K another downstream effector of Ras proteins [52, 76]. In addition, the recruitment of SH2 domain containing p85 regulatory subunit to phosphorylated RTK is also essential for the activation of PI3K [77]. Upon PI3K activation and PI(3,4,5)P<sub>3</sub> production, the protein kinases PDK1 and Akt, among numerous other PH domain containing proteins, are recruited to the plasma membrane, where these proteins control a range of signal transduction pathways [78, 79]. PDK1 displays constitutive kinase activity toward the AGC family kinases, including Akt/PKB, PKC, SGK (Serum/glucocorticoid regulated kinase) among others [79]. However, it is only when recruited to the plasma membrane that these kinase can interact with their substrates, many of which are simultaneously recruited to the plasma membrane via PH domains. Indeed, the PI3K-PDK1-Akt signaling axis is one of the most intensively studied growth/survival signaling pathways, and the simultaneous membrane recruitment of PDK1 and Akt has been shown to be essential for Akt activation [80].

### **Additional Ras Effector Pathways**

Besides MAPK and PI3K pathways, there are several other well defined Ras effector pathways. RalGDS (Ral guanine nucleotide dissociation stimulator), which functions as GEFs for the Ral GTPases, is another well-studied Ras effector [81]. Through this effector cascade, Ras can regulate the Ral GTPases, leading to the regulation of phospholipase D1 (PLD1), and Forkhead O transcription factors (FoxO) family [82, 83].

Phospholipase Cε has also been identified as a Ras effector [84]. As a member of the PLC family, PLCε hydrolyzes phosphatidylinositol 4,5-diphosphate (PI(4,5)P<sub>2</sub>),

releasing diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP<sub>3</sub>). DAG and IP<sub>3</sub> together trigger calcium release from intracellular reservoirs such as the endoplasmic reticulum (ER), and activation of protein kinase C (PKC) [84]. Therefore, Ras signaling is also able promote calcium signaling through PLC $\epsilon$ .

Ras association domain family (RASSF) proteins have recently been reported to function as direct effectors for Ras GTPases, especially K-Ras, functioning as tumor suppressors [85]. Once bound to active Ras GTPases, RASSF proteins stimulate the activity of mammalian Ste20-like kinase (MST1/2), which controls several pro-apoptotic signaling pathways [86-89]. The discovery of RASSF family as the Ras effectors reveals the “dark” side of Ras proteins, demonstrating a role for Ras signaling in promoting cell death as well as stimulating cell growth and pro-survival signaling.

Despite the complexity of the effector pathways that have been discussed above, there are still a number of Ras effector pathways, including RIN1, and AP-6, that have not been described [50, 51], and new effector pathways for the Ras family continue to be identified. The diversity of effector pathways places Ras GTPases at the center of a number of complex signaling networks within the cell, emphasizing the importance of Ras signaling and the ongoing research directed at generating a comprehensive understanding of Ras signaling.

### **Rit Subfamily GTPases**

Rit, along with Rin and the *Drosophila* ortholog Ric, comprise the Rit subfamily of Ras-related GTPases [90-93]. Rit is ubiquitously expressed, while Rin is expressed solely in neurons [90, 92]. Despite the high degree of conservation within the core structure when compared to other Ras subfamily members, Rit proteins contain some unique features which are not found in other Ras subfamily GTPases (described in more detail below), suggesting that these GTPase may control distinct physiological. Indeed, Rit has been reported to play important roles in cell transformation, neuronal differentiation, and my research supports a critical role for Rit signaling in cell survival.

### Biochemical Characteristics of Rit

Rit shares more than 50% amino acid identity with Ras, despite relatively large amino acid extensions at both N- and C- termini which account for its higher molecular weight (~25kDa) when compared to most other Ras-like G-proteins (~21kDa). Rit possesses most of the common characteristics of Ras subfamily proteins, including a high binding affinity to guanine nucleotides (GTP and GDP) as well as very low intrinsic GTPase activity [92]. However, Rit contains a number of distinct features that distinguish it from other Ras GTPases.

Unlike most of the Ras subfamily proteins, Rit lacks either C-terminal or N-terminal lipid modification signals. Instead, it contains a stretch of basic amino acid sequence within the C-terminus that is believed to encode the membrane targeting signal in Rit [21, 92]. Although these basic residues are responsible for binding to PI(3,4,5)P<sub>3</sub> on the inner leaflet of the plasma membrane, the hydrophobic residues (i.e. Trp<sup>204</sup>, Leu<sup>207</sup>, Phe<sup>211</sup>) within this polybasic cluster has been shown to be crucial for selective plasma membrane localization of Rit [21].

Rit preferentially binds to guanine nucleotide (GTP/GDP) over other nucleotides with a very low basal intrinsic GTPase activity [92]. Rit has a surprisingly rapid guanine nucleotide dissociation rate when analyzed using an *in vitro* nucleotide exchange assay [92], but the recombinant protein lacked the C-terminal poly-basic domain (to allow for expression in bacteria), which might contribute to this finding. Considering the intracellular concentration of GTP is much greater than GDP, it is conceivable that a high proportion of Rit proteins are constitutively loaded with GTP, i.e. a relatively high basal activity of endogenous Rit. However, the available data indicate that it is not the case. Only a low *in vivo* level of GTP-bound Rit is detected in serum starved cells using a bead affinity pulldown assay [92, 94]. Therefore, these *in vitro* studies do not appear to accurately represent the Rit activity in cellular environment. It is likely that the presence of regulatory proteins, such as GEFs, GAPs, and/or GDIs, contribute to these differences. As discussed previously, select point mutations within Ras GTPase core domain have been identified that either block intrinsic GTPases activity or GTP/GDP exchange, leading to constitutively active or dominant negative mutants respectively. In the case of Rit, mutation of Gln<sup>78</sup> to Leu, equivalent to the oncogenic Ras<sup>Q61L</sup> mutation, causes

complete inhibition of GTP hydrolysis, resulting in a constitutively active Rit mutant [92]. Meanwhile, the Rit<sup>S35N</sup> mutation, equivalent to Ras<sup>S17N</sup>, is predominantly GDP-bound, and has been used as a dominant-negative in a variety of cellular studies [92]. Both of these mutations have proven useful in delineating Rit GTPase-mediated signal transduction, and are used frequently in my thesis projects.

Rit, as well as Rin, have a novel effector loop (HDPTIEDAY), which has two amino acids substitution compared with Ras (YDPTIEDSY) [92]. These differences first suggested that while there might be significant overlap in the downstream pathways regulated by Rit and Ras, there might also be important differences. Based on this hypothesis, a yeast two-hybrid experiment was performed using Rit<sup>Q79L</sup> or Rit<sup>S35N</sup> as bait, and several well-characterized Ras effectors including Ras-binding domain of Raf1, the Ras interaction domain of RalGDS and Rlf, AF6, RIN1 and the p110 catalytic subunit of PI3K as prey. As predicted, Rit was found to only interact with a subgroup of these Ras effectors (i.e. Raf1-RBD, RalGDS-RID, Rlf-RID and AF6) [92]. Intriguingly, in the same yeast two hybrid experiment active Rit was not found to interact with full length A-, B-, or C-Raf [92], while more recent *in vivo* co-immunoprecipitation studies in pheochromocytoma cells (PC6) have demonstrated the active Rit preferentially associates with B-Raf [95]. These seemingly conflicting data suggest that Rit may need additional “help” by other proteins to either directly or indirectly associate with B-Raf in a GTP-dependent manner. Taken together, B-Raf is downstream of Rit signaling, but whether B-Raf functions as a true effector of Rit has yet to be determined. These results highlight some of the limitations in the methodology used to identify effector targets, and many of the putative effectors identified in the original yeast 2-hybrid assay have yet to be identified as authentic Rit effectors. However, the development new technologies, such as mass spectrometry and FRET (fluorescence resonance energy transfer), have improved the search for effector proteins [96, 97].

### **Rit-Mediated Cell Transformation**

Expression of constitutively active Rit (Rit<sup>Q79L</sup>) in NIH-3T3 cells leads to some typical transformation phenotypes [98]. First, Rit<sup>Q79L</sup> stably transfected cells display a growth advantage in culture, showing both a faster rate of proliferation and a higher

saturation density. Rit<sup>Q79L</sup> expression also allows cell to proliferate in low serum growth medium, suggesting a reduced dependency on growth factors. Rit<sup>Q79L</sup> expressing NIH-3T3 cells also acquire anchorage independent growth and are capable of forming colonies in soft agar. Moreover, injecting Rit<sup>Q79L</sup> expressing cells into nude mice results in tumor growth. All of the above support the hypothesis that Rit signaling contributes to cell transformation. However, Rit<sup>Q79L</sup>-mediated signaling alone appears insufficient to abrogate normal contact inhibition, another hallmark of cell transformation. This is supported by the observation that 3T3 cells overexpressing active Rit failed to form foci in a focus formation assay, but Rit<sup>Q79L</sup> was able to synergize with Raf22W (an active Raf1 mutant lacking the 305 N-terminal residues) or constitutively active Rho<sup>Q63L</sup> to form Rho/Rac-like foci [98]. Taken together, constitutively active mutant of Rit is weakly oncogenic. Overexpression of active Rit is sufficient to induce many but not all aspects of full cell transformation. However, more in-depth investigation of Rit-mediated signaling pathways is required to better understand the role of Rit in tumorigenesis.

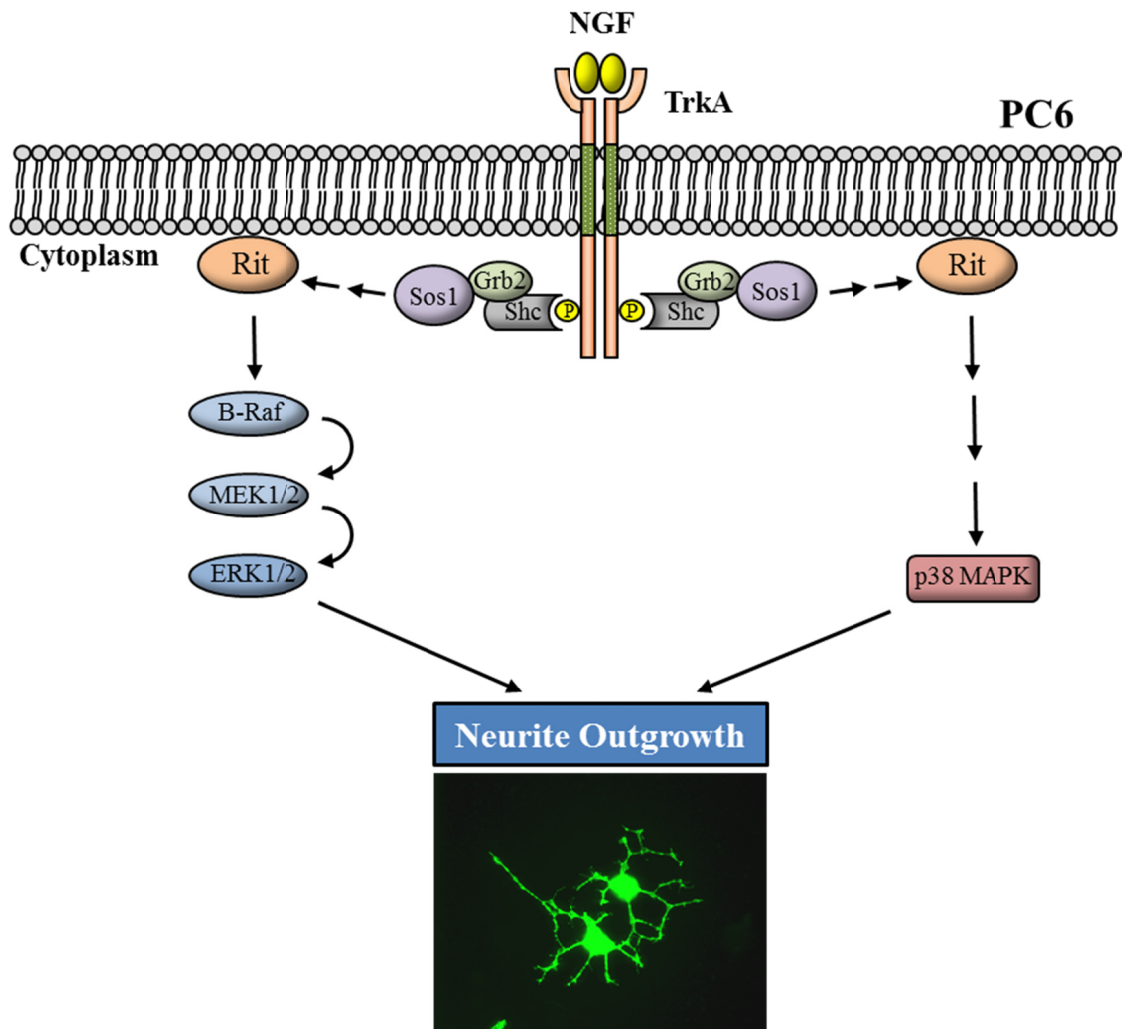
### **Rit-Mediated Neuronal Differentiation**

Unlike its ability to only moderately transform NIH-3T3 cells, constitutively active Rit is capable of potently stimulating neuritogenesis in cell lines (PC6 and SH-SY5Y cells) and regulating both axonal and dendritic morphology in primary neurons [99-101]. While other Ras family GTPases, including both Ras and the R-Ras subfamily have been previously shown to regulate neuritogenesis, the morphology of Rit-induced neuronal differentiation and the underlying mechanisms appear to be unique [2, 95]. Rit<sup>Q79L</sup> overexpression results in a highly branched neurite phenotype in PC6/PC12 cells, which is not observed in Ras<sup>Q61L</sup>-induced neurite outgrowth. The Raf/MEK/ERK signaling cascade is proven to be critical for both Ras and Rit-mediated neurite outgrowth [102]. However, recent evidence suggests these two small GTPases may not act in identical fashions. Indeed, in response to NGF stimulation, Ras appears to induce neurite outgrowth through C-Raf, while Rit preferentially acts through B-Raf, the predominant isoform in the central nervous system [95]. In addition to the ERK MAPK cascade signaling, the p38 MAPK also plays a pivotal role in Rit-mediated neuritogenesis in response to NGF [95]. Indeed, Rit appears to govern the regulation of these two kinase

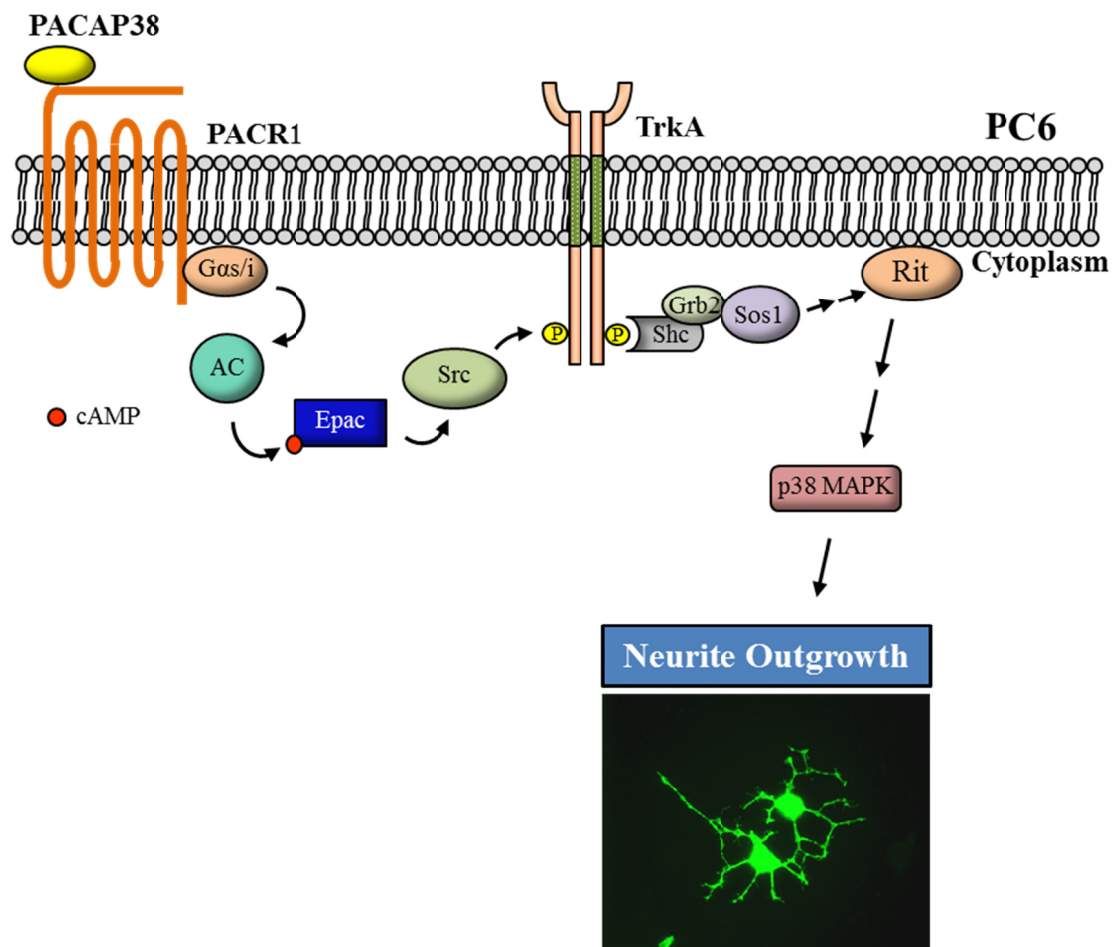


cascades to control different aspects of neuritogenesis, with the Rit/B-Raf/MEK/ERK cascade contributing to the initiation of neurite outgrowth, while Rit/p38 MAPK signaling is critical for neurite elongation and branching (Fig. 1.5) [95].

Pituitary adenylylate cyclase-activating polypeptide-38 (PACAP38) is widely expressed in the central nervous system and binds to PACAP G-protein-coupled receptors (GPCRs), to promote neuronal differentiation as well as survival [103, 104]. It has been reported that activation of adenylylate cyclase (AC) and Ras/Raf/MEK/ERK cascade following PACAP38 stimulation are the key downstream signaling pathways in regulating neuronal differentiation [105, 106]. However, the laboratorie's recent studies indicate that Rit serves as another critical small GTPase involved in PACAP38-mediated neuritogenesis [107]. As shown in Fig. 1.6, PACAP38 stimulation activates the classic GPCR/Gs $\alpha$ /AC/cAMP cascade [104]. However, PACAP38-mediated Rit activation does not require cAMP-mediated PKA activation. Instead, cAMP activates Epac, a newly indentified GEF for Rap GTPases, resulting in the activation of the non-receptor tyrosine kinase Src. The activation of Src family kinases in turn leads to the phosphorylation and activation of the TrkA receptor in a manner that bypasses the normal requirement for NGF binding [108]. Therefore, PACAP38 regulates Rit activity by transactivation of the TrkA receptor. Despite the fact that in both cases Rit is activated in a TrkA-dependent fashion, the role for Rit in PACAP38- and NGF-mediated signaling is not identical. Our studies have shown that while Rit is critical for NGF-mediated ERK activation, in the case of PACAP38 stimulation, however, Rit predominantly controls p38 MAPK to mediate neurite outgrowth [107]. These data suggest that PACAP38 dependent ERK signaling does not require TrkA receptor transactivation. Although Rit has proven important in PACAP38-mediated neuritogenesis through p38-dependent signaling, this novel pathway is not fully understood. For instance, how Epac mediates Src activation is still unclear. Despite its demonstrated role as a Rap GEF, and studies indicating that Rap is capable of stimulating Src family kinases [108], Epac activates Src in a Rap-independent fashion [107]. In addition, Src can also be activated following Gi $\alpha$  activation, through an unknown mechanism. Taken together, these data suggest that multiple signaling cascades may be involved in PACAP38-mediated Rit activation and thus neurite outgrowth.



**Figure 1.5 Rit-Mediated Neuritogenesis in Response to NGF Stimulation in PC6 Cells.** NGF binding triggers the dimerization and autophosphorylation of TrkA receptors. Active TrkA then stimulates Rit GTPase likely via the SOS GEF [108], which in turn activates both B-Raf/MEK/ERK and p38 MAPK signaling cascade to induce neurite outgrowth.



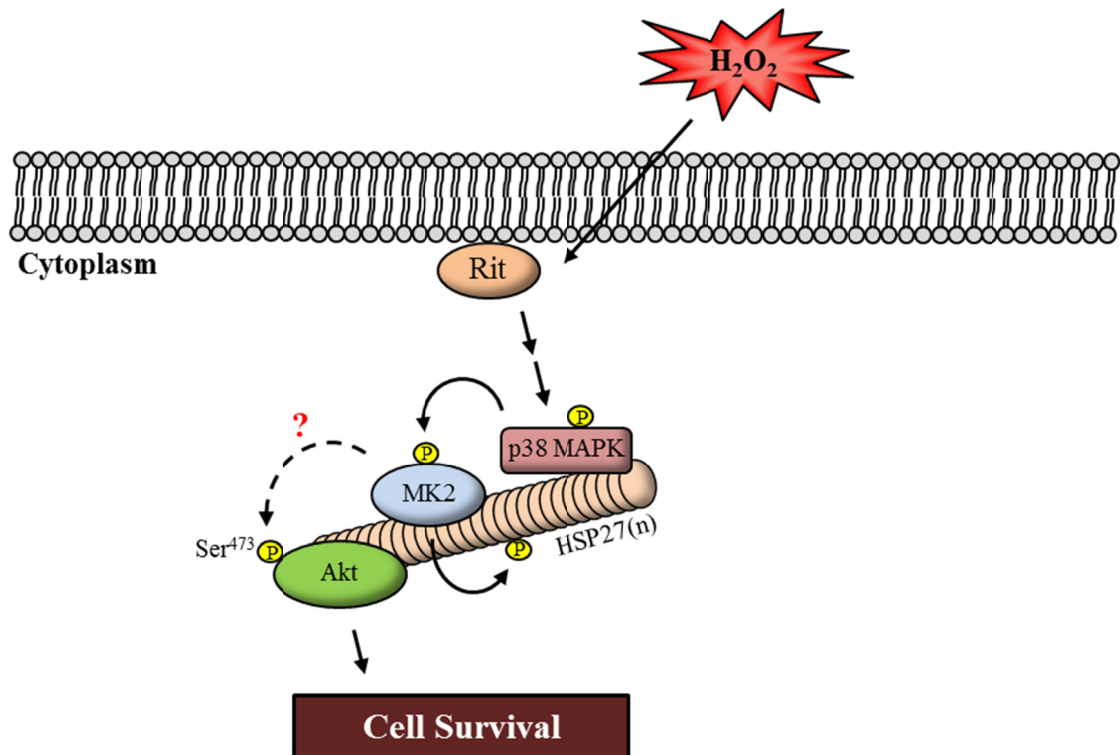
**Figure 1.6 Rit-Mediated Neuritogenesis in Response to PACAP38 in PC6 Cells.** PACAP38 binds to G-protein coupled receptor PACR1 and activates heterotrimeric G-protein  $\alpha$  subunits (G $\alpha$  and G $\alpha$ ), which stimulate cyclic AMP production by activating adenylyl cyclase (AC). cAMP binds to and activates Epac, a recently identified Rap GEF. Surprisingly, we have shown that Epac leads to the activation of Src in a Rap-independent fashion [107, 108]. Activated Src in turn leads to the transactivation of the TrkA receptor in the absence of NGF binding, and consequently triggers Rit-mediated p38 MAPK signaling to induce neurite outgrowth.

Additional studies from our lab indicate that Rit signaling also plays a central role in the control of axonal and dendritic morphology in primary neurons [109, 110]. Ectopic expression of constitutively active mutant (Rit<sup>Q79L</sup>) in the primary hippocampal neurons promotes axonal extension but inhibits dendritic elongation. Conversely, dominant negative Rit<sup>S35N</sup> overexpression blocks axonal growth while enhances dendritic elongation [109]. These opposing functions of Rit suggest that for the proper growth of both axon and dendrites, the Rit activity must be differentially regulated in these two cellular compartments. Indeed, our further studies indicate that in the sympathetic neurons, NGF activates Rit to promote axonal growth, whereas both NGF- and BMP7-mediated pathways act through suppressing Rit activation and promoting dendritic extension. In addition, MEK/ERK signaling cascade appears critical for axon-promoting and dendrite-inhibition effects of active Rit [109]. Moreover, Rit also contributes to IFN- $\gamma$ -induced dendritic retraction in a p38 MAPK-dependent fashion, further supporting the key role of Rit in regulation of neuron morphology [111].

### **Rit-Mediated Cell Survival**

Ras subfamily G-proteins are also known to play roles in cell survival [2, 25, 49]. Previous work has found that expression of either active Ras or Rit proteins elicits pro-survival signaling pathways in response to growth factor withdrawal in PC12 cells [99]. While Ras signaling had been extensively studied and has been found to promote survival by activating multiple signaling pathways including ERK and PI3K-Akt pathways [2, 25], the mechanism of Rit-mediated pro-survival signaling remains to be fully characterized. A recent study by Dr. Shi in the laboratory demonstrated that Rit signaling promotes survival not only following growth factor withdrawal, but also in response to a wide variety of cellular stresses, including DNA damaging agents (etoposide, ET), reactive oxygen species (hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>), tumor necrosis factors (TNF- $\alpha$ ), and other environmental stresses [112]. In response to these stresses, Rit, but not Ras or Rap GTPases, activates a novel p38/MK2/Akt signaling complex scaffolded by HSP27 oligomers (Fig. 1.7). Within this complex, MK2, activated by upstream Rit-p38 signaling, leads to the phosphorylation of HSP27. More importantly, this cascade appears to play a critical role specifically in the phosphorylation of Akt at Ser<sup>473</sup>, which has been

shown to be required for the maximal Akt activity [113]. Akt is known to play an important role in survival signaling (see below), and the ability of Rit to modulate Akt activity via this novel cascade appears to represent at least one important aspect of Rit-dependent pro-survival signaling. However, more studies are needed to explore how Rit is activated and coupled to p38-MK2-HSP27-Akt complex signaling in response to a diverse collection of stresses. In addition, the downstream targets of the Rit-p38-MK2-HSP27-Akt signaling cascade remain to be identified.



**Figure 1.7 Rit-Mediated Pro-Survival Signaling in Response to Cellular Stress.** Cellular stresses (e.g. H<sub>2</sub>O<sub>2</sub>) stimulate the GTP-loading of Rit GTPase, which leads to the activation of a HSP27 scaffolded signaling complex. Within this complex, p38 MAPK activates MK2, which in turn phosphorylates HSP27. While it is known that p38/MK2 activity is required for Akt phosphorylation Akt (Ser<sup>473</sup>), whether this is a direct or indirect effect, remains under investigation (see Chapter Four). When phosphorylated HSP27 triggers the dissociation of this complex and releases active Akt, which in turn activates/inactivates multiple signaling pathways to promote cell survival.

## Regulation of Akt

Akt/PKB, a serine/threonine protein kinase, plays a central role in the control of diverse cellular processes, including cell proliferation, differentiation, migration, nutrient metabolism, and programmed cell death (apoptosis) [114]. As one of the best characterized pro-survival molecules, Akt controls multiple anti-apoptotic pathways in response not only to growth factors but also to a variety of stresses (Fig. 1.8) [114]. Akt phosphorylates and either activates a number of pro-survival proteins or inactivates a variety of pro-apoptotic proteins to promote survival. For example, Akt phosphorylates Mdm2, an E3 ubiquitin ligase of p53, to downregulate p53, which controls several pro-apoptotic pathways at the transcriptional level [77, 115]. In addition, Akt phosphorylates Bad and procaspase-9, thus blocking their pro-apoptotic functions [116, 117]. Akt also provides pro-survival effects through the regulation of a number of transcription factors. For instance, Akt is known to phosphorylate and inactivate FoxO family members [117, 118]. Akt has also been shown to activate I $\kappa$ -B kinase (IKK), which phosphorylates and mediates the degradation of I $\kappa$ -B, releasing NF- $\kappa$ B from inhibitory factor (I $\kappa$ -B). This allows NF- $\kappa$ B to translocate into nucleus and induce the transcription of anti-apoptotic molecules, such as cIAP [117]. In addition, several recent studies have shown that Akt also phosphorylates and inactivates pro-apoptotic kinases (e.g. MST1/2) [119, 120].

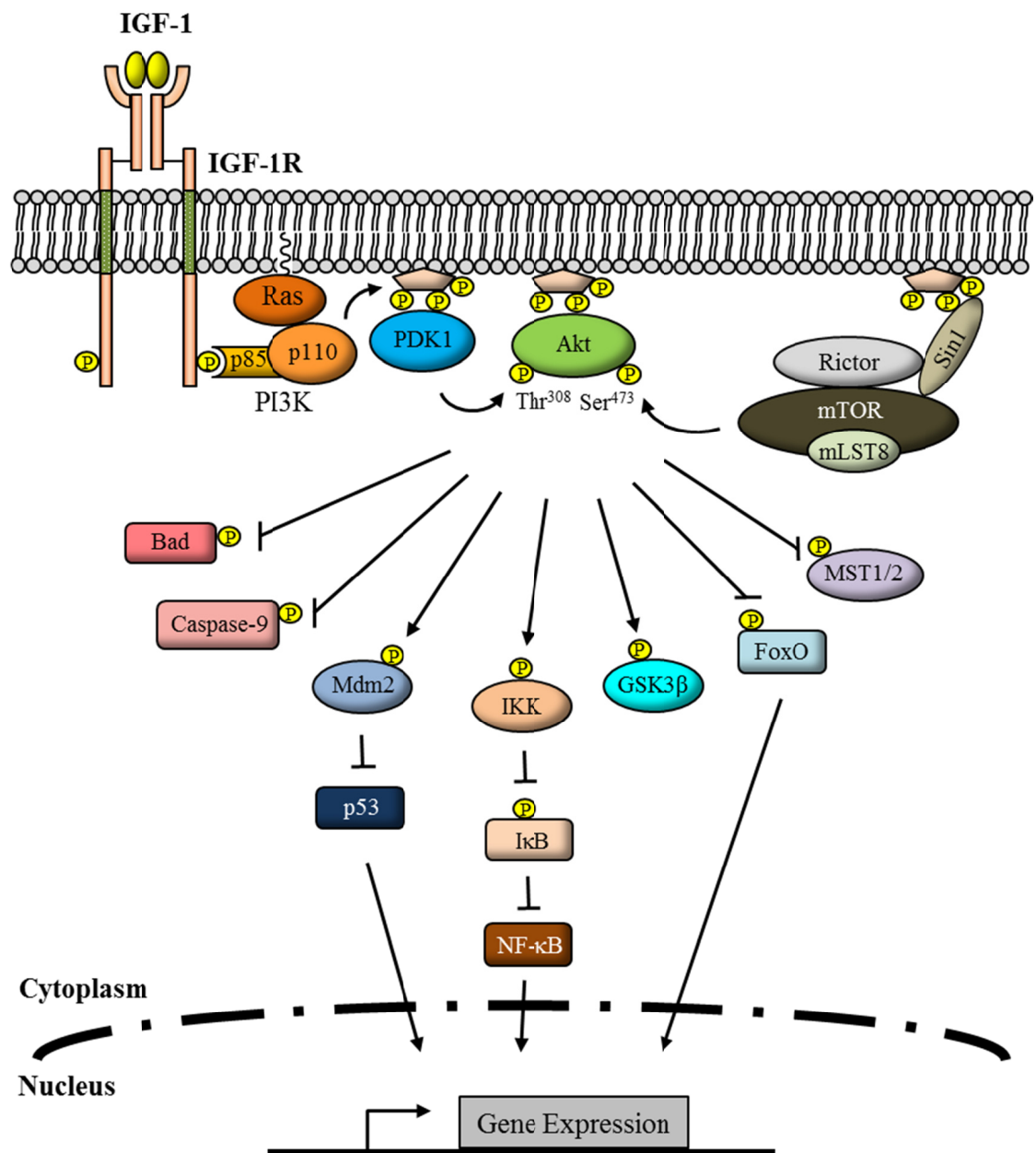
Akt activation is thought to require three steps (Fig. 1.8): membrane recruitment, phosphorylation of Thr<sup>308</sup> in the activation loop and phosphorylation of Ser<sup>473</sup> in the C-terminal hydrophobic motif. Membrane recruitment is achieved by the interaction of the pleckstrin homolog (PH) domain of Akt with the PI (3,4,5)P<sub>3</sub> and PI (3,4) P<sub>2</sub> lipid head groups, which are produced by active PI3K [80]. Membrane targeted Akt can then be activated by phosphorylation at two distinct regulatory sites. Phosphorylation at Thr<sup>308</sup> in the activation loop is essential for Akt kinase activity. This event is controlled by PDK1 kinase, which is also recruited to the PtdIns (3,4,5)P<sub>3</sub> and PtdIns (3,4) P<sub>2</sub> enriched membrane domains through its PH domain [117]. However, maximum Akt activity requires phosphorylation at Ser<sup>473</sup> within the C-terminal hydrophobic motif of Akt. This is controlled by a less well defined “PDK2” pathway [121]. To date, multiple PDK2-like pathways have been reported, of which the mammalian TOR complex 2 (mTORC2) is the best characterized [122]. mTORC2 is an evolutionarily conserved complex consisting

of mTOR, mLST8 and mTORC2 specific Rictor, Sin1 and Protor-1 proteins [123, 124]. Unlike the more widely studied mTORC1 complex, which is composed of mTOR, mLST8 and the TORC1-specific Raptor subunit [123], mTORC2 is largely resistant to rapamycin treatment, although long-term treatment of rapamycin has been reported to inhibit mTORC2 activity [125]. mTORC2 was initially reported to be essential for Akt Ser<sup>473</sup> phosphorylation in response to insulin/IGF-1 without affecting PDK1-mediated Akt phosphorylation at Thr<sup>308</sup> [122]. Further studies revealed that mTORC2 also contributed to Ser<sup>473</sup> phosphorylation in response to TGF- $\beta$  stimulation [126]. These data strongly suggest that mTORC2 is a key regulator of Akt activity. Recent studies have suggested that the p38-MK2-HSP27 complex functions as another putative Akt Ser<sup>473</sup> phosphorylation mechanism in response to H<sub>2</sub>O<sub>2</sub> exposure [127]. In addition, DNA protein kinase (DNA-PK) has been reported to directly mediate Akt Ser<sup>473</sup> phosphorylation at the DSB (double strand break) site in the nucleus following radiation damage [128]. These studies suggest that distinct cellular pathways may be utilized to control Akt activity in a stimulus dependent fashion. Our previous studies indicated that Rit contributes to Akt Ser<sup>473</sup> phosphorylation through p38-MK2-HSP27 complex in PC6 cells [112], which leads us to question whether Rit controls Akt activity via only a single cascade or is able to activate these multiple PDK2 cascades to control stress-dependent Akt activation. Chapter Four in this dissertation will address this question.

Ras family small GTPases, particularly members of the Ras subfamily, are known as a common partner and an important regulator for Akt [37]. As a physical interacting partner of class 1A PI3Ks, Ras plays a critical role in receptor tyrosine kinase-mediated PI3K/PDK1-dependent Akt phosphorylation at Thr<sup>308</sup> [117]. Indeed growth factors (e.g. VEGF, IGF-1), as well as neurotrophic factors (e.g. NGF, BDNF, GDNF), have been shown to activate Akt through PI3K/PDK1 cascade and elicit pro-survival pathways [129-132]. In addition to PI3K regulation, an evolving literature suggests that Ras subfamily members are also involved in the regulation of PDK2-like pathways. Indeed, RasC was recently shown to control TORC2-Akt pathway signaling, mediating cell migration in *Dictyostelium discoideum* [133], although in mammalian systems, the relationship between Ras GTPases and the mTORC2 complex has not yet been elucidated. In our hand, Rit does not appear to link strongly to PI3K. However, our recent



data support that Rit contributes to Akt regulation primarily through “PDK2” pathways [112]. Taken together, these evidences strongly indicate the importance of Ras subfamily proteins in Akt regulation.



**Figure 1.8 Akt Signaling Pathways.** IGF-1 stimulates IGF-1 receptor and its downstream signaling pathways including Akt. Activation of IGF-1R leads to the recruitment of PI3K and stimulates the production of PI (3,4,5)P<sub>3</sub>, which is responsible the membrane targeting of PDK1 and Akt. PDK1 then phosphorylates Akt at Thr308, while mTORC2 acts as a PDK2 pathway to phosphorylate Akt at Ser<sup>473</sup>. These two phosphorylation events result in maximum Akt kinase activity, which phosphorylates a diverse array of downstream cellular targets including pro-apoptotic protein Bad and Caspase-9, protein kinases GSK3 $\beta$  and MST1/2. Active Akt also directly or indirectly regulates transcription factors including p53, FoxO, and NF- $\kappa$ B, thereby controlling gene expression.

## **Adult Hippocampal Neurogenesis**

The vertebrate brain is one of the most complex and also vulnerable organs in the body. For a long time, it was believed that all the neurons found in the adult brain were made during embryonic development, or during a short period after birth. However, a growing body of evidence indicates that new neurons are constantly produced in restricted areas of the brain and become functionally incorporated into the mature neural network throughout adulthood [134]. This process, termed adult neurogenesis, appears to contribute to brain “plasticity” to facilitate both learning/memory and injury recovery [135], although the exact role of newborn neurons remains controversial. The two most active regions of adult neurogenesis are the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) [136-138]. Neurogenesis in the SVZ is highly active in rodents and generates neural progenitors which migrate through the rostral migratory stream to support olfactory development [136]. The functional importance of this process in human remains poorly characterized. Adult neurogenesis within the DG of the hippocampus, however, it appears to be highly active in human, and has received considerable attention.

The development of selective hippocampal X-irradiation ablation techniques and the generation of Nestin-hsvTK transgenic mice, diminishing the number of newborn neurons within the hippocampal DG, have provided the necessary experimental tools required to begin to address the physiological importance of neurogenesis [139, 140]. Irradiation results in the death of all proliferating cells within the targeted region, ablating not only proliferating neural progenitor cells but also other proliferating cells including reactive glial cells. Expression of the thymidine kinase enzyme from herpes simplex virus (hsvTK) under the control of the stem cell-specific nestin promoter drives restricted expression in neural progenitor cells in the hippocampus. Administration of the nucleotide analogue ganciclovir (GCV), results in localized phosphorylation by hsvTK, and activation of this analog only in neural progenitor cells. Once activated, GCV is incorporation into DNA during replication, resulting in the disruption of DNA replication and cell death [141]. Studies using these models have revealed an important role for adult hippocampal neurogenesis in learning and memory [140]. In particular new-born immature neurons appear to be critical for the discrimination of similar representations,

which is termed pattern separation [135, 142, 143]. More importantly, decreased adult neurogenesis not only impairs the cognitive activity in mouse models but a similar trend appears to be true in humans [140, 144]. Neurological disorders such as Alzheimer's disease, Parkinson's disease and depression, which have symptoms including cognitive deficits and mood disorders, often coincide with impaired adult neurogenesis in the hippocampus, highlighting neurogenesis as a putative therapeutic target for these neurological disorders. Indeed, some antidepressant drugs with clinical success act, at least in part, through enhancing the level of the neurogenesis in the hippocampus [145, 146].

The hippocampal dentate gyrus develops mostly during the postnatal period. A reservoir of neural stem and progenitor cells in the SGZ persist throughout adulthood and are the source of DG granule neurons [134]. These neural stem and progenitor cells can be further classified as Type 1, 2a, 2b and 3 progenitor cells [147, 148]. Type 1 cells are radial astroglial-like cells expressing both glial fibrillary acidic protein (GFAP) and nestin. Current data suggest that this population of neural progenitors serve to support self-renew of the stem cell population through symmetric division. Type 2a cells, the daughter cells from the asymmetric mitosis of Type 1 progenitors, lose the expression of GFAP while the expression of nestin is preserved. Type 2b cells are committed neural progenitor cells expressing neuronal markers such as microtubule protein doublecortin (Dcx) and polysialylated neural cell adhesion molecule (PSA-NCAM), along with nestin. Type 3 cells are late committed neural progenitors, lacking nestin expression. Dcx expressing committed neural progenitors (Type 2b and 3) are also called new-born immature neurons. They begin to develop short pre-mature dendritic and axonal projections, display hypersensitivity to neurotransmitters, and gradually migrate toward the granular layer of the DG. Within 4-6 weeks new-born immature neurons mature and become fully integrated into the neural circuitry of the DG. Mature granule cells express mature neuron marker Neuronal Nuclei (NeuN), while the expression of both Dcx and PSA-NCAM is lost [147, 148].

The rate of adult neurogenesis in the DG is not constant, but dynamically regulated [149-152]. Homeostasis appears to be controlled by two different mechanisms: the generation of committed neural progenitors from stem cells and the survival and

maturation of newborn immature neurons [150, 153-155]. Both of these processes appear to be tightly monitored and dynamically regulated. For instance, physical exercises have been shown to stimulate the production of committed neural progenitors [150], while environmental enrichment predominantly promotes the survival of newborn immature neurons and facilitates the functional integration of these new neurons into the hippocampal circuitry [156]. Despite the well accepted concept that increases in both physical and cognitive activity induce adult neurogenesis, the molecular mechanisms underlying this phenomenon remain poorly understood. A growing body of evidence suggests that neurotrophins (BDNF, NGF, and NT-3) as well as growth factors (IGF-1, bFGF and VEGF) may play important roles in adult neurogenesis [157-162]. Among them, IGF-1 and BDNF have been most intensively studied. Locomotion activity increases the peripheral IGF-1 which can cross the blood brain barrier to interact with IGF-1R receptors in the brain, including in the hippocampus. As a result, local IGF-1 concentration within the hippocampus is elevated after physical exercise [163]. Interestingly, elevated IGF-1 signaling coincides with the enhanced proliferation of neural progenitor cells, and this increase is blocked by administration of anti-IGF-1 antibody, implicating an essential role for IGF-1 in the stimulation of exercise-induced adult neurogenesis [164]. However, the downstream signaling pathways from IGF-1R remain unclear. Several pathways, including ERK MAPK signaling and the PI3K-Akt-GSK3 $\beta$  cascade, have been proposed to be critical in IGF-1-induced proliferation of neural progenitor cells [165-167]. It is possible that multiple signaling cascades acts cooperatively to promote neurogenesis in response to IGF-1. Studies have also shown that increases in BDNF also positively correlate with both adult hippocampal neurogenesis and cognitive performance [157]. For instance, physical exercise and enriched environment both result in elevated BDNF levels and increased adult neurogenesis in the hippocampus [168]. Despite the finding that BDNF appears to have the same overall effect on adult neurogenesis as IGF-1, the mechanism of BDNF-regulated neurogenesis is still under debate. Conditional knock-out of TrkB (the receptor for BDNF) in hippocampal neural progenitor cells, which blocks BDNF signaling, significantly impairs proliferation and neurogenesis in DG, supporting a critical role for BDNF in neural progenitor cell proliferation [169]. However, studies in transgenic mice

with either aberrant BDNF levels (BDNF<sup>+/-</sup>) or dominant negative TrkB overexpression suggest that BDNF may, in fact, be required for the survival of newborn immature neurons without affecting the proliferation of neural progenitor cells [170]. These seemingly conflicting conclusions may be explained by inherent differences in the various mouse models. Alternatively, it is possible that BDNF signaling is required for both proliferation and cell survival during neurogenesis. Additional investigations are needed before any definitive conclusions can be drawn.

The hippocampus is sensitive to a large array of brain injuries/stresses including hypoxia, seizure, and brain trauma [171-173]. Neural stem cells and new born immature neurons within the DG appear to be the most fragile, with much of the observed cell loss occurring in these two populations [173, 174]. Recent work has shown that even modest traumatic brain injury results in a massive loss of neural stem cells and immature neurons immediately after the initial insult, followed by an increase in neural progenitor proliferation and the generation of new born immature neurons often days or weeks after the initial injury [165, 173, 175, 176]. This induction of adult neurogenesis appears to help replenish the neural progenitor pool and may partially compensate for the neuronal loss after injury. The dynamic modulation of neurogenesis following brain injury raises two equally important questions. First, what is the molecular mechanism of initial neuron loss following injury? Decoding the corresponding signaling pathways may provide therapeutic strategies to prevent the excessive neuron loss following brain injury before it is too severe to repair. Most of these injuries cause a combination of cellular stresses which directly leads to neuron degeneration. These cellular stresses include reactive oxygen species (ROS), excitotoxicity, and excessive inflammatory factors, among others [177-179]. Therefore, therapies designed to either block stress-induced apoptotic pathways or enhance endogenous protective mechanisms may ameliorate the neuron loss observed following injury. Conditional knockout of BDNF in the hippocampal DG results in increased newborn immature neuron loss following traumatic brain injury (TBI), suggesting that BDNF signaling is such a putative therapeutic target [174]. Second, what triggers the induction of neurogenesis after injury? Newborn neurons successfully incorporated into neural circuitry appear to help hippocampal recovery. Several groups have reported that the upregulation of adult neurogenesis following

traumatic brain injury and stroke is critical for cognitive recovery [180-182]. A collection of neurotrophins and growth factors have been proved to promote neurogenesis in both physiological and pathophysiological conditions. For instance, IGF-1 is elevated following status epilepticus promoting neural progenitor cell proliferation in an ERK-dependent fashion [165]. Therefore, it is of great clinical interest to explore the mechanisms underlying enhanced neurogenesis. A greater understanding of this mechanism(s) might allow manipulation, an approach which is already being used to target IGF-1 signaling, to facilitate neural recovery.

### **Rationale for Investigating the Physiological Functions of Rit and Their Mechanisms**

As a molecular switch, Rit, an orphan Ras family GTPase, is activated by a diverse array of extra- and intra-cellular stimuli. Previous studies indicate that Rit may be involved in multiple biological processes, including cell proliferation and transformation, neuronal differentiation and morphogenesis, and cell survival [95, 98, 99, 107, 109]. However, these studies were all performed in *in vitro* cultured cell models, which may not accurately reflect physiological conditions. This is particularly true since Rit and Ras share both closely related effector domains, and at least some downstream effector pathways in common [2, 49, 100]. Indeed, expression of activated dRit in *Drosophila* results in morphological changes typically associated with Ras signaling [93], but knockout of *dRit* failed to alter the development/differentiation of these same tissues [183], suggesting that overexpression of an activated Rit/RIC mutant may not provide a clear picture of its true physiological function. Based on this concern, we generated a genetically engineered Rit null mouse line in which Rit gene expression is lost throughout the body. With this Rit<sup>-/-</sup> mouse model, we are capable of investigating the physiological role of Rit. In Chapter Three, we examine the role of Rit to the signaling pathways activated by a diverse collection of extracellular stimuli including growth factors and cellular stresses in primary mouse embryonic fibroblasts (MEFs). These studies suggest the provocative notion that a key physiological role for Rit is to promote cell survival following oxidative stress. It appears p38-MK2-HSP27-Akt signaling pathway is one of the Rit-mediated pro-survival mechanism. However, it was unclear



whether Rit only controls this pathway or multiple mechanisms to regulate Akt activity. Therefore, in Chapter Four, we investigate the role of mTORC2 complex, as a PDK2 pathway, in H<sub>2</sub>O<sub>2</sub>- and Rit-mediated Akt phosphorylation. Our studies suggest that mTORC2 can physically interact with Rit GTPase. The kinase activity of mTORC2 appears to be dependent on p38 MAPK, indicating the Rit-p38 signaling as upstream regulator of mTORC2. Collectively, these studies have broader implications not only in general cell survival mechanisms following stress but also in pathology such as the role of HSP27 in Charot-Marie-Tooth disease. In Chapter Five, we demonstrate that Rit is also critical in neuronal survival, especially in newborn immature neurons within the hippocampus, following oxidative stress as well as in response to traumatic brain injury. Furthermore, in Chapter Six, we examine the role of Rit in IGF-1 signaling particularly in neurons. The dependency of IGF-1 mediated signaling on Rit indicates a key role of Rit in IGF-1 mediated adult neurogenesis. As reviewed above, adult neurogenesis has been proven key in both the normal physiology such as neuron population refreshment and learning/memory and the recovery from brain injuries and the resultant cognitive disorders. The identification of these Rit-mediated signaling could raise some clinical insights in the treatment or recovery of brain injuries such as brain trauma and also neurodegenerative disorders.

## CHAPTER TWO

### Materials and Methods

#### A. General Methods

**Plasmids and Reagents** — Flag-tagged wild-type, constitutively active, and dominant negative Rit and H-Ras in p3×FLAG-CMV-10 vector (Sigma, Saint Louis, MO), GFP-tagged Rit and H-Ras in pEGFP-C1 vector (Clontech, Mountain View, CA), HA-tagged Rit and K-Ras in pKH3 vector, Glutathione-S-Transferase (GST)-tagged Rit and Akt in pEBG vector (kindly provided by J. H. Kehrl, National Institute of Allergy and Infectious Diseases, National Institutes of Health), RGL3-Rit Binding Domain (RBD) in pGEX-KG vector (kindly provided by S. W. Whiteheart, Department of Molecular and Cellular Biochemistry, University of Kentucky) were generated by polymerase chain reaction (PCR) amplification and verified by sequence analysis.

Myc-tagged human Rictor, mTOR and Raptor cDNA clones were the kind gifts of Dr. Tianyan Gao, Department of Molecular and Cellular Biochemistry, L. P. Markey Cancer Center, University of Kentucky. Rat Sin1 cDNA clone was purchased from Open Biosystems and subcloned into the pCMV-Myc vector (Clontech) and pCMV5-GFP vector.

Lentivirus packaging vector pSPAX2, lentivirus envelop vector pMD2.G, and lentiviral shRNA expressing vector containing shRNA sequences for mTORC1/2 components (pLKO.1-shCTR, shRictor, shSin1, shRaptor) were also the kind gifts of Dr. Tianyan Gao (Markey Cancer Center, Univ. Kentucky). Lentivirus was produced by the Department Molecular and Cellular Biochemistry Viral Core under the direction of Dr. Michael Mendenhall.

The following commercially available antibodies were used: Flag,  $\beta$ -actin (Sigma); Myc; GFP (NeuroMab, UC Davis); GST (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); phospho-p38, p38, phospho-ERK1/2, ERK1/2, phospho-ERK5, phospho-Akt (Ser<sup>473</sup> and Thr<sup>308</sup>), Akt, phospho-MK2 (Thr<sup>334</sup>), phospho-HSP27 (Ser<sup>82</sup>), phospho-Bad (Ser<sup>136</sup>), Bad, Rictor, mTOR, Raptor (Cell Signaling); HSP27, Sin1 (CalBiochem); Doublecortin (Dcx), BrdU (Abcam); Neuronal Nuclei (NeuN) (Millipore); Nestin (Covance), MAP2 (Clone AP20, Sigma); FITC-conjugated anti-mouse IgG, FITC-

conjugated anti-rabbit IgG, Cy3-conjugated anti-rat IgG, Cy5-conjugated anti-mouse IgG (JacksonImmuno).

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and tunicamycin (TM) (Sigma); etoposide (ET) (CalBiochem); EGF, bFGF, IGF-1 (R & D systems); Inactive His-tagged Akt (Full length) (Millipore); kinase specific inhibitors SB203580 (Tocris), PD98059 (CalBiochem); Hoechst 33258 (Sigma); Fluorojade c (Millipore) were purchased from the indicated vendors.

**Cell Culture and Transfection** — Mouse embryonic fibroblasts (MEFs) and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone), 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. HEK-293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 5% heat-inactivated fetal bovine serum (FBS, HyClone), 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. All the cells were cultured at 37 °C in a humidified atmosphere of 5%  $\text{CO}_2$ .

MEFs and HeLa cells were transfected using Superfect (Qiagen) or Transgen (Apharma) according to the manufacturer's protocol. Briefly,  $4 \times 10^5$  cells were seeded into each well of the six-well plate the day before transfection. On the day of transfection, the cells should be 70-80% confluent. Transfection DNA-lipid complex was prepared by mixing 1.5  $\mu\text{g}$  plasmid DNA with 5  $\mu\text{L}$  of Transgen/Superfect transfection reagent in 150  $\mu\text{L}$  Opti-MEM (Gibco) and incubated at room temperature for 20 min. The DNA-lipid complex was then dropped to the cell monolayer in 1 mL Opti-MEM medium. After 4 h incubation, the cells were placed into the fresh culture medium for another 48hr before further experiments.

HEK-293T cells were transfected by  $\text{Ca}^{2+}$ /Phosphate method. Briefly,  $9 \times 10^5$  HEK-293T cells were seeded into each well of the six-well plate the day before transfection. On the day of transfection, HBS/phosphate mixture was generated by combining 1 mL of  $2 \times$  HBS and 20  $\mu\text{L}$  of  $100 \times$  phosphate solution. 3  $\mu\text{g}$  total DNA plasmids are diluted in  $\text{dH}_2\text{O}$  to a total volume of 94  $\mu\text{L}$ . 6.4  $\mu\text{L}$  2 M  $\text{CaCl}_2$  was added and mixed well. The DNA mixture (100  $\mu\text{L}$ ) was then added drop-wise to 100  $\mu\text{L}$  HBS/phosphate solution with gentle mixing followed by 30 min incubation at room

temperature. A total of 200  $\mu$ L transfection complex was added drop-wise to the HEK-293T cell monolayer in 1 mL fresh culture medium and incubated overnight. The cells were re-fed with 2 mL fresh culture medium after the incubation and cultured for another 48 h before further experiments.

### **Statistical Analysis**

All data were presented as mean  $\pm$  S.D., unless otherwise noted. Student's *t*-test was used to analyze the differences between the individual experiments.

### **B. Rit Mediates a Pro-Survival Pathway in Response to Reactive Oxygen Species**

**Generation of Rit Knockout Mice** — Mice were housed in a pathogen-free facility and handled in accordance with standard use protocols, animal welfare regulations, and the NIH *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee. A targeting vector (Fig. 3.1A) containing approximately 5.7 kb of the murine *Rit1* gene was constructed from 129/Sv strain mouse genomic DNA by the Gene Targeted Mouse Service Core at the University of Cincinnati, replacing the coding region of exon 2 with the lacZ gene together with a neomycin cassette (with flanking FRT sites). The targeting vector was transfected into ES cells derived from 129S6/SvEv Tac mice and correctly recombined G418-resistant clones were identified by PCR and Southern blot analysis. Two independent ES clones were injected into blastocysts and male mice with a high degree of chimerism were crossed to wild-type Black Swiss females to generate *Rit*<sup>+/-</sup> mice. Heterozygous offspring were mated to generate homozygous *Rit*<sup>-/-</sup> mice.

**Mouse Genomic DNA Extraction and Genotyping PCR** — Genomic DNA was extracted from tail-snips by incubation in tail lysis buffer (100 mM Tris-HCl pH 8.8, 5 mM EDTA, 0.2% SDS and 200 mM NaCl) containing 0.4 mg/mL proteinase K (Invitrogen) at 55 °C overnight, followed by incubation with 60  $\mu$ g/ml RNase (Invitrogen) at 37 °C for 1 h. DNA was precipitated, and resuspended in 10 mM Tris-HCl pH 8.0 prior to genotyping analysis. Primers used for *Rit* genotyping were as follows:  
Wild-type allele:

Forward primer: 5'-GTGAAGGCCGAGGATGTAGG-3' (oligo a)

Reverse primer: 5'-GGTCATGGTCTTCTGGGAATCG-3' (oligo c)

Knock-out allele:

Forward primer: 5'-ACCCGTGATATTGCTGAAGAGC-3' (oligo b)

Reverse primer: 5'-GGTCATGGTCTTCTGGGAATCG-3' (oligo c)

The PCR parameters were  $T_m = 53\text{ }^{\circ}\text{C}$  for 35 cycles. The PCR products were 462 bp for wild-type allele; 347 bp for knock-out allele (See Fig. 3.1C).

**Reverse Transcription-PCR Analysis** — To determine whether expression of Rit was eliminated in Rit-knockout mice, RT-PCR was performed. Briefly, total RNA was isolated with TRIzol reagent (Invitrogen) from Rit<sup>+/+</sup>, Rit<sup>+/-</sup> and Rit<sup>-/-</sup> MEF cultures. Total RNA (2  $\mu\text{g}$ ) was subjected to reverse transcription using the Enhanced Avian HS RT-PCR kit (Sigma), and the levels of Rit expression were then examined by PCR using first strand cDNA as template. GAPDH served as a control. The primer pairs were:

Mouse Rit forward: 5'-CAATGCAGTTCATCAGCCACC-3'

Mouse Rit reverse: 5'-CGTGGAAAACGTCGTCGATG-3'

Mouse GAPDH forward: 5'-AAGCCCATCACCATCTTCCAG-3'

Mouse GAPDH reverse: 5'-AGGGGCCATCCACAGTCTTCT-3'

The PCR parameters were  $T_m = 53\text{ }^{\circ}\text{C}$  for 30 cycles (See Fig. 3.1D).

**Primary MEF Isolation and Maintenance** — Primary MEFs were isolated from E13.5 embryos following standard protocols. Briefly, timed pregnant mice (either wild-type or Rit<sup>-/-</sup> mice) at day 13.5 post coitum were euthanized. The uterine horns were dissected out, rinsed with  $1\times$  PBS briefly and placed into a petri dish containing  $1\times$  PBS. Individual embryos were separated from the placenta and the surrounding membrane. The brain, the dark red organs (mostly liver and spleen), as well as the tubular intestine were removed and saved for DNA extraction and genotyping. Each embryo was numbered and transferred into a 15 mL conical tube containing 1 mL 0.25% Trypsin-EDTA (Invitrogen) and 1 kU DNase I (Sigma). Cells were triturated in DMEM plus 10% FBS following 30 min digestion at  $37\text{ }^{\circ}\text{C}$ . The dissociated cells were washed once with DMEM plus 10% FBS and plated onto 10 cm dishes (1 embryo/dish). The MEFs were maintained in

DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin and split 1 to 5 once the monolayer reached confluence. All MEF experiments were performed using cells at passage 3 to 5 unless otherwise noted.

**Immortalization of Primary MEFs** — The day before infection with a retrovirus expressing SV40 large T-antigen (the kind gift of Dr. Andrei V. Budanov, UCSD),  $3 \times 10^5$  primary MEFs (passage 2) were seeded in 60 mm dishes and cultured overnight. On the day of infection, 1.5 ml of virus supernatant (SV40 large T-antigen) supplemented with polybrene (10 µg/ml) (Sigma) was applied to MEFs for 3.5 h, after which monolayers were washed and returned to culture medium. 24 h later, a second round of infection was performed, and immortalized MEFs selected by continuous passage until a stable population doubling time was obtained. The cells were then maintained in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. To generate MEFs stably expressing GFP or GFP-Rit<sup>Q79L</sup>, immortalized MEFs were transfected and subjected to G418 (1 mg/ml) selection, single colonies were picked and expanded. GFP and GFP-Rit<sup>Q79L</sup> expression was confirmed by western blot (Fig. 3.3A).

**Protein Phosphorylation Analysis** — Whole cell lysates were prepared using kinase lysis buffer [20 mM Hepes (pH 7.4), 150 mM NaCl, 50 mM KF, 50 mM β-glycerolphosphate, 2 mM EGTA (pH 8.0), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 10% glycerol, and 1× protease inhibitor cocktail] and subsequently subjected to experimental analysis. Protein phosphorylation was determined by immunoblotting with the appropriate phospho-specific antibodies and band intensity quantified by Image J (NIH).

**Rit-GTP Loading Assays** — GST fusion proteins containing the Rit binding domain (RBD) of RGL3 (residues 610-709) were used to precipitate GTP-loaded Rit as described previously [95, 107]. Briefly, immortalized cells were transfected (Superfect, Qiagen) with 3×Flag-Rit-WT (1 µg) and serum starved prior to stimulation. The cells were then subjected to a variety of stimuli, harvested at the indicated times, and total cell lysates prepared. Total protein (400 µg) was subjected to GST-RGL3-RBD agarose pull-down with end-over-end rotation at 4 °C for 2 h. The pelleted GST-RGL3-RBD agarose resin

was subjected to extensive washing, and GTP-Rit levels (active Rit) were determined by immunoblotting with anti-Flag monoclonal antibody.

**Apoptosis Analysis** — Cell apoptosis was examined by examining caspase-3 and PARP cleavage or nuclear condensation [184]. The levels of caspase-3 and PARP cleavage were determined by immunoblotting total cell lysates with anti-caspase-3 and anti-PARP antibodies [112].

Nuclear condensation was analyzed by Hoechst staining. Briefly, primary Rit<sup>+/+</sup> or Rit<sup>-/-</sup> MEF cultures were left untreated or subjected to indicated dosage of H<sub>2</sub>O<sub>2</sub> for the indicated time. After treatment, cells were fixed with fresh 4% paraformaldehyde (PFA) for 15 min, rinsed three times with PBS, incubated in 1 µg/mL Hoechst 33258 (Sigma) for 10 min to label nuclei, and washed three times with PBS. Cells were examined for condensed and fragmented nuclei and representative images were captured using a Zeiss Axiovert 200M fluorescence microscope (32× objective).

A non-radioactive cell proliferation assay (MTS) (Promega) was used to determine cell viability. Briefly, primary Rit<sup>+/+</sup> or Rit<sup>-/-</sup> MEFs were plated in 96-well plates at a density of 2,000 cell/well and allowed to recover for 24 h. On the day of the experiment, cells were left untreated or subjected to the indicated dose of H<sub>2</sub>O<sub>2</sub> for indicated period of time. For the inhibitor studies, cultures were incubated with 10 µM SB203580 for 30 min prior to the H<sub>2</sub>O<sub>2</sub> treatment. Following H<sub>2</sub>O<sub>2</sub> exposure, the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI) was used to monitor metabolic activity according to manufacturer's protocol by measuring absorbance at 490 nm.

### **C. Rit-Mediated Akt<sup>Ser473</sup> Phosphorylation Requires mTORC2 Complex**

**Lentivirus Infection** — HeLa cells and HEK-293T cells were seeded onto 60cm dishes at a density of 8×10<sup>5</sup> and 2×10<sup>6</sup> per mL, respectively, 24 h before transduction. Lentivirus (5 µL) containing shRNA sequences targeting human Rictor, Sin1, or Raptor were applied to the cells along with 10 µg/mL polybrene (Sigma). After 5 h incubation, cells were re-fed with fresh culture medium and incubated overnight. On the second day, puromycin was added to the culture medium to a final concentration of 2 µg/mL to enrich

for the infected cells. After 7 days of drug selection, knock-down efficiency was determined by Western blot. Cells were used in experiments for no more than one month.

**UV Irradiation** — MEFs were seeded onto 35 cm dishes at a density of  $8 \times 10^5$  per mL the day before UV irradiation. MEFs were serum-starved for 3 h and washed once with  $1 \times$  PBS prior to UV exposure ( $50 \text{ mJ/cm}^2$ ) without any medium or PBS in the dish. After exposure, cells were re-fed with fresh serum-free medium and incubated for another 30 min. Total cell lysates were prepared using kinase lysis buffer [20 mM Hepes (pH 7.4), 150 mM NaCl, 50 mM KF, 50 mM  $\beta$ -glycerolphosphate, 2 mM EGTA (pH 8.0), 1 mM  $\text{Na}_3\text{VO}_4$ , 1% Triton X-100, 10% glycerol, and  $1 \times$  protease inhibitor cocktail] and subsequently subjected to immunoblotting.

**Co-Immunoprecipitation Analysis** — To examine protein interactions by co-immunoprecipitation (Co-IP), cell lysates were prepared from HEK-293T cells transiently co-transfected with the indicated pair of epitope-tagged protein expression vectors. Cells were allowed to recover for 48 h after transfection and then harvested using kinase lysis buffer. Protein lysates (400  $\mu\text{g}$ ) were incubated with the indicated antibodies (2  $\mu\text{g}$ ) in a total volume of 800  $\mu\text{L}$  for 1 h at 4 °C with end-to-end rotation. Immunocomplexes were affinity absorbed onto 15  $\mu\text{L}$  of protein G-sepharose beads (GE Healthcare) for an additional hour at 4 °C with end-to-end rotation. Sepharose resin was then washed 1 time with kinase lysis buffer, 2 times with kinase lysis buffer supplemented with 500 mM NaCl, and finally times with kinase lysis buffer. Bound proteins were eluted by incubation for 5 min at 100 °C in SDS loading buffer. Bound proteins along with total cell lysates (25  $\mu\text{g}$ ) from each sample were resolved using 10% SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblotting using the indicated antibodies.

**mTORC2 Complex Kinase Assay** — To examine the kinase activity of the mTORC2 complex, we performed the *in vitro* kinase assay using inactive His-Akt (Millipore) as substrate [125]. HeLa cells with appropriate treatment were harvested using mTORC2 lysis buffer [40 mM Hepes (pH 7.4), 120 mM NaCl, 50 mM KF, 10 mM  $\beta$ -



glycerolphosphate, 1 mM EDTA (pH 8.0), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.3% CHAPS, and 1× protease inhibitor cocktail]. Protein lysates (1 mg) were incubated with anti-Rictor antibodies (1 µg, Bethyl Laboratory) in a total volume of 600 µL for 90 min at 4 °C with end-to-end rotation. Immunocomplexes were affinity absorbed with protein G-sepharose beads (15 µL) (GE Healthcare) for an additional hour at 4 °C with end-to-end rotation. Sepharose resin was washed 4 times with mTORC2 kinase lysis buffer, followed by 2 washes with mTORC2 kinase buffer [25 mM Hepes (pH 7.4), 100 mM KAc, and 1 mM MgCl<sub>2</sub>]. TORC2 kinase reactions were performed by incubating the immunoprecipitates in kinase assay reaction mix (15 µL) [TORC2 kinase buffer with 200 ng inactive His-Akt and 200 µM ATP] for 30 min at 37 °C. To terminate the reaction, 135 µL of kinase buffer and 50 µL of 4×SDS were added to the reaction and mixed prior to incubation for 5 min at 95 °C. 20 µL of each samples were loaded onto the 10% SDS-PAGE for immunoblotting analysis. Kinase activity was monitored by immunoblotting with phospho-Akt (Ser<sup>473</sup>) specific antibody. The equal loading of individual proteins was confirmed by immunoblotting with indicated antibodies.

#### **D. Rit Promotes Selective Immature Neuron Survival following Oxidative Stress and Traumatic Brain Injury**

**Primary Hippocampal Neuron Culture from Post-natal Mouse Pups** — Primary cultures of hippocampal neurons were prepared from C57BL6 wild-type and Rit<sup>-/-</sup> pups within 12 h of birth (P1). Hippocampi were surgically dissected from the brain and then dissociated by treatment for 30 min at 37 °C in a solution of DMEM supplemented with 4 mg/mL papain (Sigma) and 0.0025 mg/mL DNase I (Sigma). The tissues were then triturated in DMEM plus 10% FBS and plated at a density of 5×10<sup>5</sup> cells per 35 mm poly-D-lysine (0.1 mg/mL in 5 mM Tris pH 8.0) coated tissue culture dishes or equivalent density in other ploy-D-lysine coated dishes or on coverslips. After a 4 hr incubation at 37°C, the medium was replaced with serum-free Neurobasal medium (Invitrogen) supplemented with 0.5 mM glutamine (Invitrogen), B27 (Invitrogen) and 100 µg/mL streptomycin and 100 U/mL penicillin (Invitrogen) (Complete Neurobasal medium). Cells were incubated in a humidified incubator at 37°C and 5% CO<sub>2</sub>. After 3 days *in vitro* (DIV3), half of the growth medium was removed and replaced with fresh

complete neurobasal media supplemented with 2  $\mu$ M AraC to prevent the proliferation of non-neuronal cells (astrocytes and glial cells). At DIV7, the cells were re-fed with complete neurobasal media. All the experiments were performed at DIV8 unless otherwise noted.

**Immunostaining and Apoptosis Assay** — At DIV8, the hippocampal neuron cultures were left untreated or subjected to indicated dosage of H<sub>2</sub>O<sub>2</sub> for indicated time at 37 °C. Cells were fixed with fresh 4% paraformaldehyde (PFA) for 15 min. To identify neuronal cells, immunostaining was performed with indicated antibodies. Briefly, cultures fixed with 4% PFA were washed three times with PBS and permeabilized by 5 min incubation in 1×PBS containing 0.1% Triton X-100 at room temperature (RT). Cultures were blocked for 2 h at RT in 5% normal goat serum (NGS), incubated overnight at 4 °C with indicated primary antibodies diluted in 1×PBS containing 5% NGS and 0.1% Triton X-100. MAP-2 antibody (1:1000) was used to label neuronal dendrites and cell bodies. Dcx (1:1000) was used to label immature neurons. NeuN (1:1000) was used to label mature neurons. Nestin (1:200) was used to label neural stem cells (NSC). Then the cultures were washed three times with 1×PBS, and incubate overnight at 4 °C with indicated fluorochrome-conjugated goat secondary antibodies (FITC anti-Mouse IgG, 1:1000; Rhodamine anti-Rabbit IgG, 1:1000) diluted in 1×PBS containing 5% NGS and 0.1% Triton X-100. The cultures were rinsed three times with 1×PBS and rinsed three times with 1×PBS and coverslipped with Slowfade Gold antifade mounting media with DAPI (Invitrogen). Cells were examined for condensed and fragmented nuclei using a Zeiss Axiovert 200M fluorescence microscope and representative images were captured with an Orca ER camera using a 32× objective.

**Controlled Cortical Impact (CCI)** — Surgeries were performed by Shaun W. Carlson in Dr. Kathy E. Saatman's lab in ScoBIRC, Department of Physiology, University of Kentucky. The surgical procedures were performed as described previously [185].

**BrdU Administration** — 11-week old mice were intraperitoneally (i.p.) injected with 3-bromodeoxyuridine (BrdU, 50 mg/kg in 0.9% saline, Sigma) daily for one week. After

the final injection, mice were housed for another two days prior to CCI in order to deplete any remaining unincorporated BrdU.

**Tissue Preparation** — This work was done by Shaun W. Carlson and Jennifer M. Brelsfoard in Dr. Kathryn E. Saatman's lab in ScoBIRC, Department of Physiology, University of Kentucky.

**Histology** — The morphological analysis of hippocampus  $Rit^{-/-}$  mice were achieved by either Nissl or DAPI staining. Briefly, serial coronal sections (40  $\mu$ m thick) were cut and collected in to 5 vials. Half of the sections from each vial (400  $\mu$ m intervals between sections) were mounted onto Fisher Superfrost Plus slides, stained for Nissl or DAPI, and coverslipped with Vectashield. Representative images for Nissl staining were taken using an AX80 light microscope. DAPI images were taken using a Zeiss Axiovert 200M fluorescence microscope. To assess the average thickness of the dentate gyrus, images of the dentate gyrus from 3 consecutive sections within the group (400  $\mu$ m intervals) at the epicenter of the hippocampus or equivalent depth were chosen. The area and the length of the dentate gyrus were measured by Image J (NIH) and the average thickness was obtained by dividing the area of the dentate gyrus by the length for the three chosen sections.

**Fluorograde C Staining** — Serial brain sections (40  $\mu$ m thick, 400  $\mu$ m intervals) were soaked in 1 $\times$ PBS for 5 min before mounting onto Fisher Superfrost Plus slides. The slides were then dried on a 40-45  $^{\circ}$ C slide warmer for 15 min followed by overnight air drying. On the second day, sections were rinsed in 80% (v/v) ethanol containing 1% (w/v) NaOH, 70% (v/v) ethanol, and dH<sub>2</sub>O sequentially prior to incubation in 0.06% (w/v) potassium permanganate for 10 min in the dark and five subsequent dH<sub>2</sub>O washes. The sections were then incubated in 0.0001% Fluorograde C solution (1 mL 0.01% FJC to 99 mL of 0.1% acetic acid) in the dark for another 10 min followed by extensive washing with dH<sub>2</sub>O. DAPI was used as a counterstain agent to label cell nuclei. The slides were dried, dehydrated with xylene, coverslipped with Cytoseal and kept at 4  $^{\circ}$ C in the dark before analysis using a fluorescence microscope. To quantify FJC-positive cells, three

consecutive sections (pre-epicenter, epicenter, and post-epicenter, 400  $\mu$ m intervals) from each animal were counted. The sums of the three sections are presented for each animal. Representative images were captured using an Olympus CKX31 A1 confocal microscope (20 $\times$  objective).

**Immunofluorescence Analysis** — The brain sections (40  $\mu$ m thick, 400  $\mu$ m intervals) were placed in 24-well plates (up to 3 sections per well) and washed three times in 1 $\times$ PBS for 5 min. The sections were then permeablized and blocked by the addition of 5% normal goat serum (NGS) and 0.1% Triton X-100 in 1 $\times$ PBS by placement on a rotary shaker for 30 min at room temperature. After blocking, primary antibodies in a cocktail at the appropriate concentration diluted in 5% NGS in PBS/TritonX (0.1%) were applied to the brain sections, and subjected to gentle rocking on a shaker platform overnight at 4 °C. On the following day, the sections were washed 4 times in 1 $\times$ PBS and a secondary antibody cocktail diluted in 5% NGS in PBS/TritonX (0.1%) was applied. The sections were then washed 4 times with 1 $\times$ PBS, mounted on Fisher Superfrost Plus slides, coverslipped with SlowFade Gold anti-fade reagent containing DAPI, and sealed with nail polish. The slides were stored at -20 °C before further analysis.

For BrdU detection extra steps were required to further denature the genomic chromatin in order to allow access of the anti-BrdU antibody. For this analysis, prior to permeablization and blockade by NGS, sections were incubated in 2 N HCl at room temperature for 30 min, followed by the application of neutralizing buffer (0.1 M Borate buffer pH 8.0) for 10 min, and 3 times PBS washes. To improve overall image quality, one overnight PBS wash was included.

The dilutions of the antibody cocktails were:

Rabbit anti-Dcx:	1:500
Mouse anti-NeuN:	1:1000
Rat anti-BrdU:	1:500
Goat anti-Rabbit IgG FTIC:	1:1000
Goat anti-Mouse IgG Cy5:	1:1000
Goat anti-Rat IgG Cy3:	1:1000

To quantify the number of fluorescently labeled cells in the dentate gyrus, three consecutive sections (pre-epicenter, epicenter, post-epicenter, 400  $\mu$ m intervals) from each animal were counted using a Nikon Eclipse E600 fluorescence microscope (40 $\times$  objective). Since each section is much thicker than the focal plane, the focal plane was moved up and down to focus throughout the section to capture every positive cell. To estimate the volume of the dentate gyrus, images of the dentate gyrus were shot using a Zeiss Axiovert 200M fluorescence microscope (10 $\times$  objective). The volume of the dentate gyrus was then calculated by multiplying the area of the dentate gyrus which was measured using Image J (NIH) by the thickness of each section (40  $\mu$ m). Cell density was obtained by dividing the total cell counts by the total volume of the dentate gyrus for the three sections that were counted. Representative images were captured using an Olympus CKX31 A1 confocal microscope (20 $\times$  objective).

#### **E. Rit Mediates IGF-1 signaling**

**Protein Phosphorylation Analysis** — Primary wild-type and Rit<sup>-/-</sup> MFEs were serum starved for 3 h prior to 100 ng/mL EGF, bFGF or IGF-1 for 10 min. The total cell lysates were prepared using kinase lysis buffer [20 mM Hepes (pH 7.4), 150 mM NaCl, 50 mM KF, 50 mM  $\beta$ -glycerolphosphate, 2 mM EGTA (pH 8.0), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 10% glycerol, and 1 $\times$  protease inhibitor cocktail] and subsequently subjected to experimental analysis. Protein phosphorylation was determined by immunoblotting with the appropriate phospho-specific antibodies. For the primary hippocampal neurons, wild-type and Rit<sup>-/-</sup> (DIV8) were briefly starved with serum free DMEM for 40 min prior to 100 ng/mL IGF-1 or 50 ng/mL BDNF stimulation for 10 min. The total cell lysates were prepared using kinase lysis buffer and subsequently subjected to experimental analysis. Protein phosphorylation was determined by immunoblotting with indicated antibodies.

## **CHAPTER THREE**

### **Rit Mediates a Pro-Survival Pathway in Response to Reactive Oxygen Species**

#### **INTRODUCTION**

Cells are constantly exposed to a variety of environmental stresses, including genotoxic stress, endoplasmic reticulum (ER) stress, and oxidative stress, and have evolved diverse protective mechanisms. For instance, ATM/ATR signaling is known to mediate the DNA repair process in response to DNA damage [186]. Chaperone proteins facilitate the refolding of denatured proteins, and proteins that fail to refold will eventually undergo ubiquitin-proteasome system-mediated degradation (UPS) [187, 188]. For aerobic organisms, exposure to reactive oxygen species (ROS) cannot be avoided since they are generated as intermediate products of intracellular metabolism. However, antioxidants such as Glutathione, Superoxide dismutase (SOD) and catalase, etc. are capable of monitoring the level of intracellular ROS and eliminating the excessive ROS as needed [189]. The production of ROS beyond the cell's antioxidant capacity, however, causes oxidative stress and leads to cellular damages, and eventually cell death. Indeed, excessive ROS is known to contribute to the pathogenesis of numerous human diseases [190-192]. Therefore, the ability to adapt to oxidative stress is critical for cell and tissue survival.

ROS-activated signaling mechanisms have evolved to promote cell survival and homeostasis in response to oxidative damage [193, 194]. Hydrogen peroxide exposure activates all of the conventional mitogen-activated protein kinases (MAPKs) including ERK1/2, ERK5, p38 MAPK as well as JNK1/2. Among these MAPKs, ERK1/2 and ERK5 are usually associated with cell proliferation and survival, while JNK1/2 is mostly considered pro-apoptotic in response to an array of cellular stresses [193]. However, p38 MAPK signaling is quite complex, as both pro-apoptotic and pro-survival effects have been reported downstream of activated p38, operating in a context-specific manner [195-197]. All these MAPKs act by directly phosphorylating downstream targets/substrates, but also by regulating the activity of a group of downstream kinases [198], which in turn regulate additional cellular targets such as transcription factors [199]. Ultimately, MAPKs are capable of governing a wide range of signaling pathways. The balance

between the duration and magnitude of these MAPK pathways appears key to determining cell fate. However, how these MAPK signaling cascades are coordinated to determine whether the cell should undergo apoptosis or recovery in response to oxidative stress remains incompletely understood.

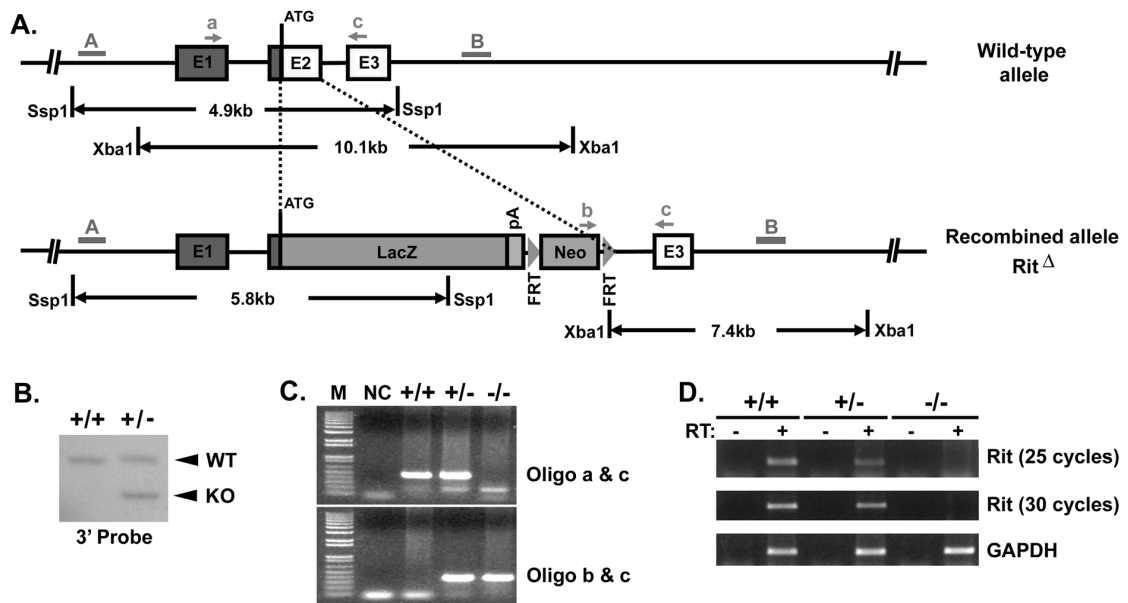
Ras-related small GTPases function as molecular switches to control a wide variety of biological processes by regulating diverse effector pathways, including MAPK and Akt pathways. As a member of Ras family GTPases, Rit is known as a critical regulator in proliferation, neuronal differentiation as well as survival in cell line studies [98, 99, 109, 112]. However, the principle physiological role of Rit remains to be characterized. In this chapter, using a genetic approach, we identify a fundamental role of Rit GTPase in ROS-activated MAPK regulation and pro-survival signaling. Loss of endogenous Rit renders primary embryonic fibroblasts susceptible to apoptosis. The pro-survival effect of Rit is at least in part due to its ability to activate a p38-dependent MK2-HSP27-Akt signaling cascade.

## RESULTS

### Generation of Rit<sup>-/-</sup> Mice

To examine the physiological role of Rit signaling, we generated a *Rit1* knockout mouse by replacing the coding region of exon 2 of the Rit gene with the lacZ gene together with a neomycin cassette (with flanking FRT sites) (Fig. 3.1A). The successful knock-out of Rit was proved at the genomic DNA level (Southern blot, Fig. 3.1B, genotyping PCR, Fig. 3.1C) and at the mRNA level (reverse transcription-PCR, Fig. 3.1D). Unfortunately, we were unable to confirm protein loss, we lack the high affinity anti-Rit antibody needed to detect endogenous Rit.

Rit null mutant mice were born at the expected Mendelian ratio and grew to adulthood without showing any discernible abnormalities. Examination of various tissues did not reveal any gross morphological or anatomic abnormalities suggesting that Rit protein is not essential for proliferation or embryonic development.



**Figure 3.1 Rit Knock-out Mice.** (A) Schematic representation of the strategy used to generate the Rit knockout mouse. Part of Exon 2 was targeted and replaced by a LacZ expressing cassette, followed by the neomycin gene flanked by FRT sites. The DNA probe (3' Probe) used for Southern analysis is indicated by the grey bar, while arrows indicate the location of genotyping primers (a, b and c). (B) Genomic DNA isolated from heterozygous Rit (Rit<sup>+/-</sup>) or wild-type (WT) embryonic stem (ES) cells were subjected to Xba I digestion, and the resulting genomic DNA fragments were resolved by agarose gel electrophoresis and transferred to nylon membrane. The Rit genomic locus was detected by Southern blot using the radiolabeled 3' Probe. As illustrated in *panel A*, WT-ES cells are expected to contain a 10.1 kb fragment, while a 7.4 kb fragment should be detected from Rit<sup>+/-</sup> ES cells. (C) Genomic DNA was isolated from mouse tail biopsies, and subjected to PCR analysis using the oligonucleotide primer pairs indicated in *panel A*. A 347 base pair band represents the mutant allele (*lower panel*; amplification using primer pair b/c), while a 462 base pair band represents the wild-type allele (*upper panel*; amplification using primer pair a/c). (D) Total RNA was isolated from cultured embryonic fibroblasts obtained from Rit mutants or WT-littermates, and subjected to RT-PCR analysis. GAPDH was included as a load control. Note that Rit mRNA levels are completely eliminated in Rit<sup>-/-</sup> MEFs, while reduced in Rit<sup>+/-</sup> MEFs.



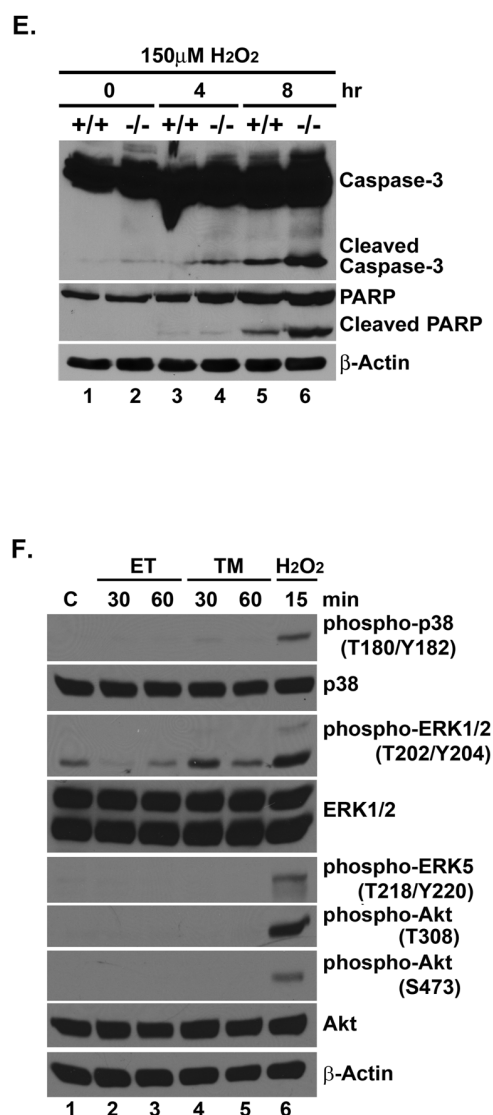
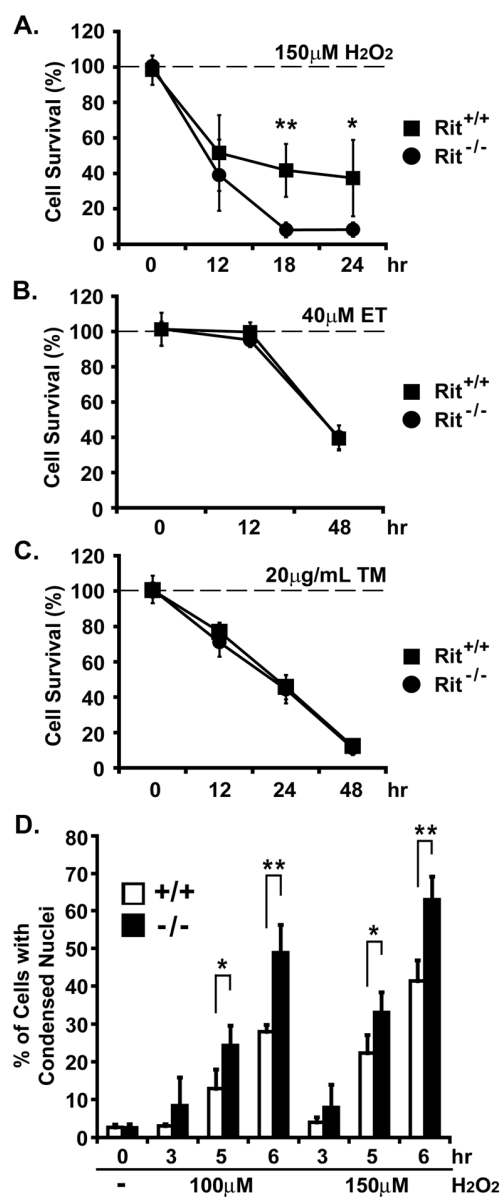
### Selective Vulnerability to Oxidative Stress in Rit<sup>-/-</sup> Fibroblasts

To evaluate cell survival, primary day 13.5 mouse embryonic fibroblasts (MEFs) were cultured from both Rit null and wild-type littermates. These cultures were left untreated or were exposed to a variety of cellular stresses including ROS (150  $\mu$ M hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>), DNA damaging agent (40  $\mu$ M etoposide, ET), or ER stress (20  $\mu$ g/ml tunicamycin, TM). Cell numbers were analyzed over time using the MTS assay, and cell viability decreased in a time-dependent manner with each of the stresses (Fig. 3.2A-C). However, hydrogen peroxide exposure resulted in a dramatic reduction in the number of Rit null fibroblasts compared to similarly stressed wild-type cells (18 h exposure: 41.8%  $\pm$  14.9% and 8.2%  $\pm$  4.2% wild-type and Rit<sup>-/-</sup>, respectively,  $p < 0.001$ ) (Fig. 3.2A). Apoptosis was increased in hydrogen peroxide-treated Rit null MEFs as monitored by the presence of condensed nuclei (Fig. 3.2D) and cleaved caspase-3 and PARP (Fig. 3.2E). This was not the case for cells exposed to either etoposide (48 h exposure: 39.4%  $\pm$  4.1% and 40.2%  $\pm$  3.3% wild-type and Rit<sup>-/-</sup>, respectively,  $p > 0.6$ ) (Fig. 3.2B) or tunicamycin (24 h exposure: 45.8%  $\pm$  6.9% and 44.6%  $\pm$  7.9% wild-type and Rit<sup>-/-</sup>, respectively,  $p > 0.7$ ) (Fig. 3.2C), in which both wild-type and Rit null fibroblasts displayed an equivalent reduction in cell viability.

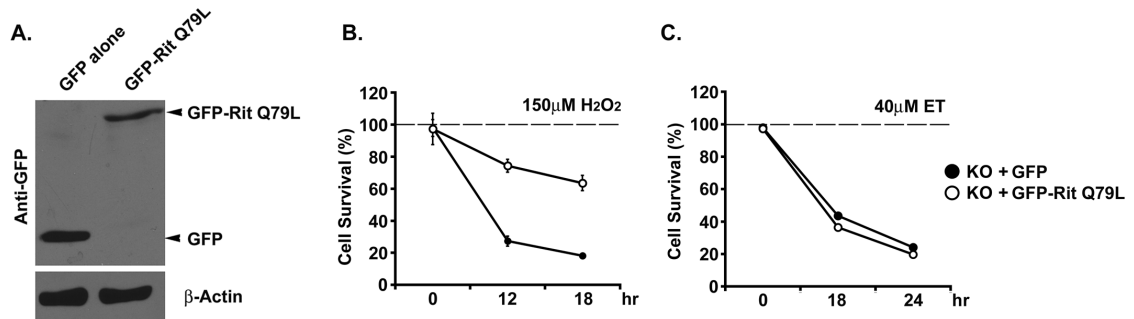
Analysis of the MAPK signaling in wild-type MEFs following hydrogen peroxide (100  $\mu$ M) treatment resulted in activation of p38, ERK, and ERK5. In addition, Akt was found to be activated in response to ROS stimulation (Fig. 3.2F). In contrast, exposure to etoposide or tunicamycin failed to stimulate p38, ERK5, and Akt signaling in wild-type MEFs (Fig. 3.2F), and ERK was only modestly activated by tunicamycin, suggesting that Rit-mediated regulation of one or more of these pathways might contribute to cell survival in response to oxidative stress.

To confirm that the survival defect was the result of Rit loss, we established stable Rit<sup>-/-</sup> MEFs, immortalized with SV40 large T antigen, expressing either green fluorescent protein (GFP) or GFP-tagged Rit<sup>Q79L</sup>, a constitutively active Rit mutant (Fig. 3.3A). Exposure of GFP control cells to hydrogen peroxide (150  $\mu$ M) induced robust cell death, while cells expressing active Rit (Rit<sup>Q79L</sup>) were significantly protected (12 h exposure: 10.9%  $\pm$  2.4% and 36%  $\pm$  1.9% GFP and GFP-Rit<sup>Q79L</sup>, respectively,  $p < 0.001$ ) (Fig. 3.3B). In contrast, Rit<sup>Q79L</sup> expression had only a modest effect on cell survival following

etoposide (40  $\mu$ M, ET) exposure (Fig. 3.3C). These data support the notion that Rit regulates an oxidative stress survival mechanism.



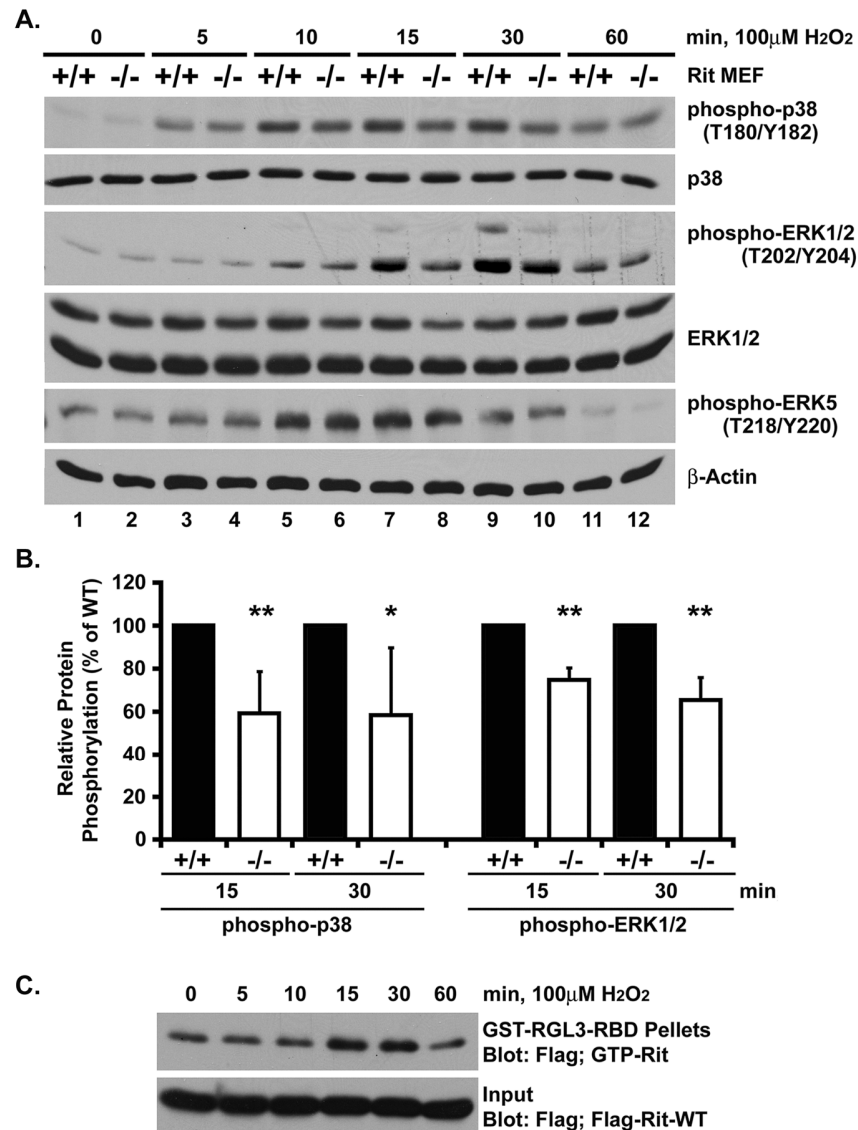
**Figure 3.2 Loss of Rit Sensitizes MEFs to Oxidative Stress but not DNA Damage and ER Stress.** (A-C) Wild-type (Rit<sup>+/+</sup>) and Rit<sup>-/-</sup> MEFs were treated with H<sub>2</sub>O<sub>2</sub> (150  $\mu$ M for 12, 18 and 24 h), etoposide (40  $\mu$ M for 12, 24 and 48 h) and tunicamycin (20  $\mu$ g/mL for 12, 24 and 36 h). Cell viability was determined by MTS metabolism at indicated times after initial exposure of these stresses. The results are presented as mean  $\pm$  S.D. (*t*-test; \* *p* < 0.05, \*\* *p* < 0.01, *n* = 3). (D) Wild-type (Rit<sup>+/+</sup>) and Rit<sup>-/-</sup> MEFs were treated with H<sub>2</sub>O<sub>2</sub> for indicated dosages and durations, fixed and stained with Hoechst to visualize nuclear morphology. The apoptotic cells were determined by condensed or fragmented nuclei. The results are presented as mean  $\pm$  S.D. (*t*-test: \* *p* < 0.05, \*\* *p* < 0.01, *n* = 4). At least 300 cells were scored for each data point. (E) Wild-type (Rit<sup>+/+</sup>) and Rit<sup>-/-</sup> MEFs were first treated with H<sub>2</sub>O<sub>2</sub> (150  $\mu$ M) for indicated times. Total cell lysates were harvested and immunoblotted with caspase-3, PARP, and  $\beta$ -actin antibodies. (F) Wild-type MEFs were exposed with etoposide (ET, 40  $\mu$ M), tunicamycin (TM, 20  $\mu$ g/ml) and H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) exposure for the indicated times after 3 h serum-free starvation. The total cell lysates were then prepared and analyzed by immunoblotting with the indicated antibodies.



**Figure 3.3  $Rit^{-/-}$  MEFs Expressing Constitutively Active Rit Are Protected from Reactive Oxygen Species.** (A) Total cell lysates from immortalized  $Rit^{-/-}$  MEFs stably transfected with GFP alone or GFP- $Rit^{Q79L}$  were prepared and immunoblotted with GFP and  $\beta$ -Actin antibodies. (B-C) GFP- and GFP- $Rit^{Q79L}$ -overexpressing  $Rit^{-/-}$  MEFs were treated with  $H_2O_2$  (150  $\mu$ M for 12 and 18 h) and etoposide (40  $\mu$ M for 18 and 24 h). Cell viability was determined by MTS metabolism at indicated times after initial exposure of these stresses. The results are presented as mean  $\pm$  S.D. ( $t$ -test; \*  $p < 0.05$ , \*\*  $p < 0.01$ ,  $n = 3$ ).

### **Rit Regulates ROS-Mediated MAPK Activation**

We next assessed the contribution of Rit to ROS-mediated intracellular signaling. Stimulation of serum-starved wild-type MEFs with hydrogen peroxide led to the activation of p38, ERK, and ERK5 MAPK kinase cascades, while ROS-dependent p38 and ERK pathway activation was significantly reduced in Rit<sup>-/-</sup> mutant cells (Fig 3.4A, B) (15 min exposure: 59.2% ± 19.6% and 74.8% ± 5.6% for p38 and ERK, respectively, n = 4, p < 0.01). Importantly, Rit does not participate in all ROS-mediated MAPK signaling as ERK5 activation was not altered in mutant fibroblasts. Since Ras-related GTPases respond to extracellular stimuli by exchanging GTP for bound GDP [2], we next tested whether hydrogen peroxide exposure triggered Rit activation. Rit-GTP loading studies are technically difficult in primary cells because of low levels of endogenous Rit and the lack of high affinity anti-Rit antibodies. Thus, we performed Rit-GTP pull-down assays using MEFs transiently transfected with 3×Flag-Rit-WT as described previously [95, 109]. Stimulation with hydrogen peroxide resulted in a transient increase in GTP-bound Rit levels (Fig. 3.4C), with activation kinetics consistent with a role for Rit in ROS-dependent p38 activation.

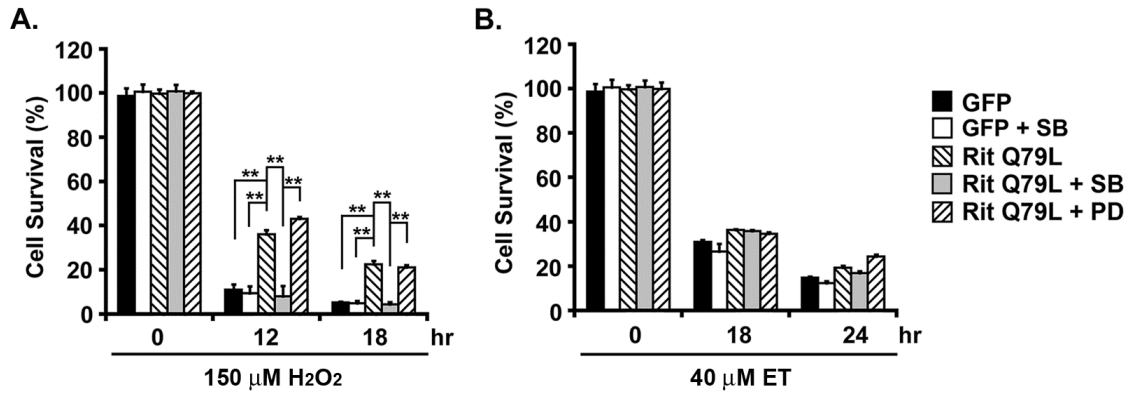


**Figure 3.4 Rit Selectively Controls MAPK Signaling following ROS.** (A) Lysates from wild-type and Rit<sup>-/-</sup> MEFs were prepared following H<sub>2</sub>O<sub>2</sub> exposure (100  $\mu$ M) for the indicated times and analyzed by immunoblotting with the indicated antibodies. (B) The relative phosphorylation of p38 and ERK1/2 after 15 and 30 min of H<sub>2</sub>O<sub>2</sub> exposure was compared between wild-type (black bars) and Rit<sup>-/-</sup> (white bars) MEFs. The results are presented as mean  $\pm$  S.D. (*t*-test: \* *p* < 0.05, \*\* *p* < 0.01, *n*  $\geq$  4). (C) Immortalized wild-type MEFs transfected with Flag-Rit-WT were serum starved for 3 h, prior to stimulation with H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) for the indicated times, and GTP-bound Rit recovered by GST-RGL3-RBD pull down. Precipitated GTP-Rit was detected by anti-Flag immunoblotting.

### **Rit-Mediated Survival Requires p38 MAPK**

The impaired phosphorylation of ERK1/2 and p38 MAPK in Rit null MEFs following H<sub>2</sub>O<sub>2</sub> exposure prompted us to examine the contribution of these pathways to survival. To address this question, we used pharmacological inhibitors specific for MEK/ERK cascade and p38 MAPK signaling on Rit<sup>-/-</sup> MEFs stably overexpressing the GFP-Rit<sup>Q79L</sup> fusion protein. While pharmacological blockade of MEK/ERK signaling (10  $\mu$ M PD98059) had no effect on Rit<sup>Q79L</sup>-dependent survival following hydrogen-peroxide exposure, p38 inhibition (10  $\mu$ M SB203580) almost completely blocked the survival advantage afforded by Rit signaling (12 h exposure: 43%  $\pm$  0.9% and 8%  $\pm$  4.7% PD and SB, respectively,  $p < 0.001$ ) (Fig. 3.5A). As expected, these same inhibitors had no effect on cell survival following etoposide treatment in MEFs expressing GFP alone or GFP-Rit<sup>Q79L</sup> (Fig. 3.5B). Taken together these data suggest that p38 signaling is a central feature of Rit-dependent survival signaling.



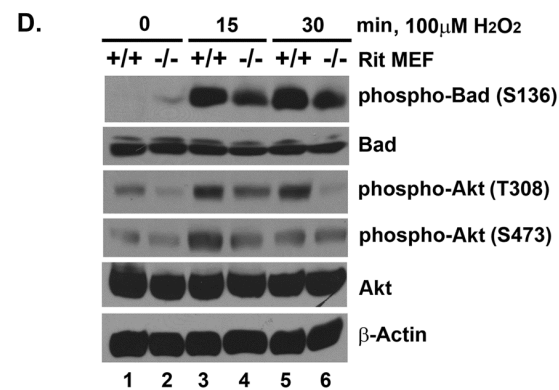
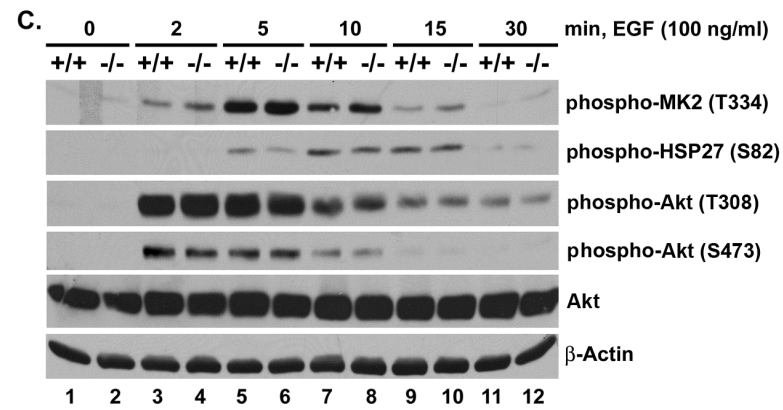
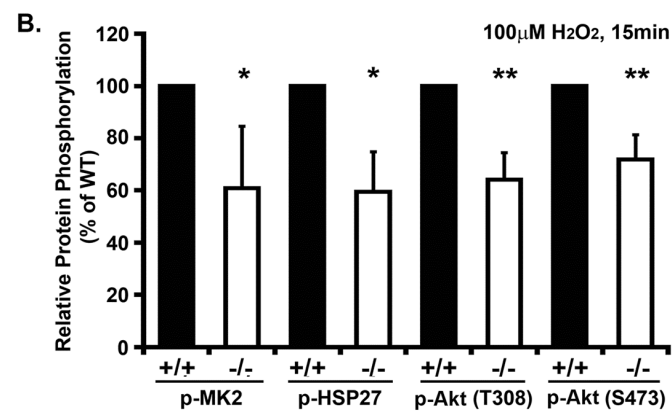
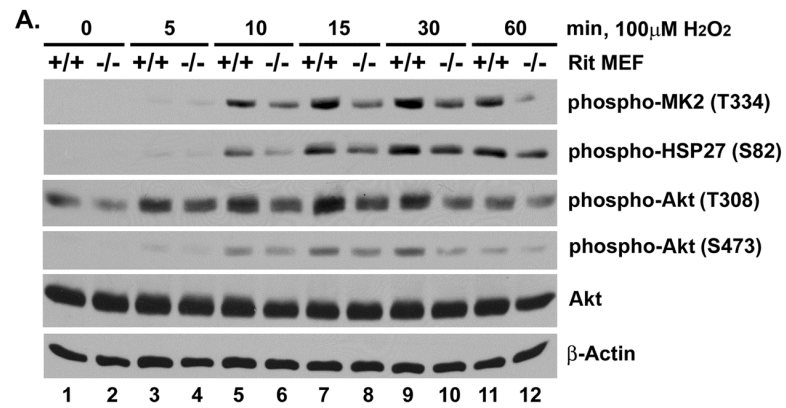


**Figure 3.5 p38 MAPK Activity Is Required for Rit-Mediated Protection from Oxidative Stress.** (A-B) GFP- and GFP-Rit<sup>Q79L</sup>-overexpressing Rit<sup>-/-</sup> MEFs were treated with  $H_2O_2$  (150  $\mu$ M for 12 and 18 h) and etoposide (40  $\mu$ M for 18 and 24 h) in the presence or absence of the p38 inhibitor SB203580 (SB, 10  $\mu$ M) or MEK inhibitor PD98059 (PD, 10  $\mu$ M). Cell viability was determined by MTS metabolism at indicated times after initial exposure of these stresses. The results are presented as mean  $\pm$  S.D. (*t*-test; \*\*  $p < 0.01$ ,  $n = 3$ ).

### **Rit-Dependent Survival Pathway Involves p38-Mediated Akt Signaling**

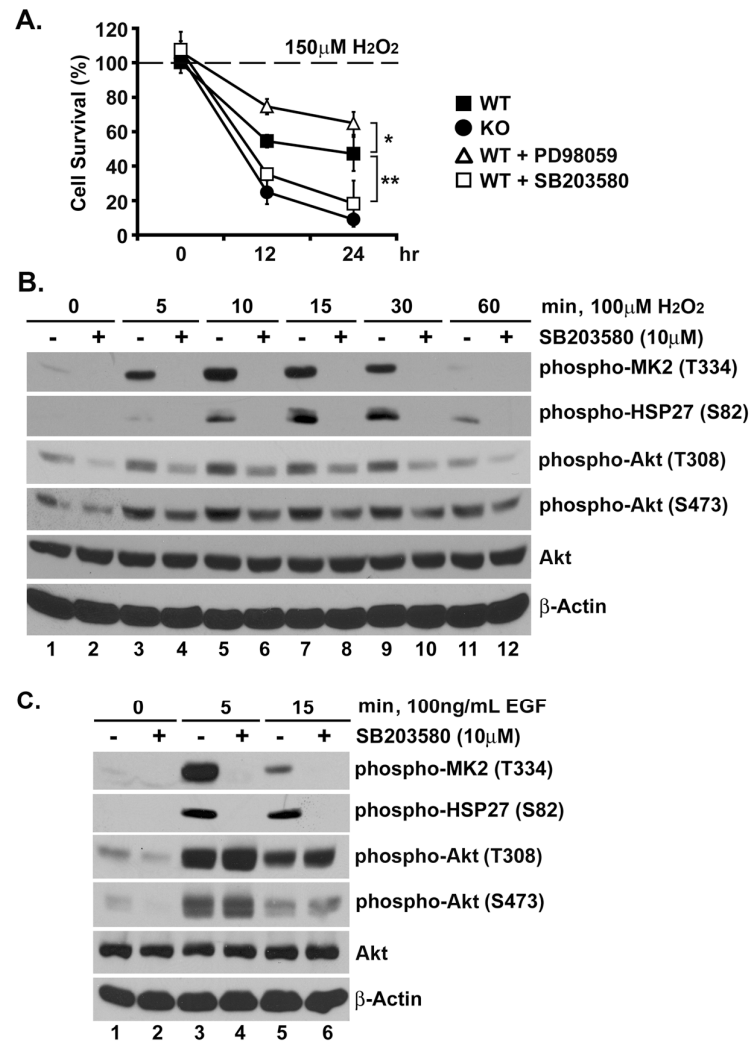
While stress-mediated p38 activation is commonly associated with cell death induction, recent work has identified a survival cascade in which p38 promotes MK2 kinase activation within a novel HSP27 scaffolded complex. This allows MK2 to phosphorylate targets including HSP27 and Akt, leading to cellular responses including the inhibition of apoptosis [200-202]. Using RNAi approaches, we have recently found that Rit contributes to stress-mediated regulation of this cascade in cultured cells [203]. Consistent with a role for Rit in the regulation of this cascade in primary MEFs, MK2 (15 min exposure, 60.8%  $\pm$  23.8%,  $p < 0.05$ ), HSP27 (15 min exposure, 59.5%  $\pm$  15.5%,  $p < 0.05$ ), and Akt phosphorylation (15 min exposure, 64.2%  $\pm$  10.3% and 71.9%  $\pm$  9.4% for Thr<sup>308</sup> and Ser<sup>473</sup> respectively,  $p < 0.01$ ), were decreased in Rit<sup>-/-</sup> MEFs following hydrogen peroxide exposure, when compared to similarly treated wild-type cells (Fig. 3.6A,B). In contrast, loss of endogenous Rit has no effect on MK2, HSP27 and Akt phosphorylation upon EGF stimulation (Fig. 3.6C). These results suggest that Rit selectively couples to p38-MK2-HSP27-Akt signaling cascade in response to oxidative stress but not growth factors.

Bad, a pro-apoptotic member of the Bcl-2 family is regulated by Akt, which specifically phosphorylates Bad at Ser<sup>136</sup> [204, 205] resulting in the sequestration of phospho-Bad in the cytosol. This prevents Bad from associating with mitochondria where it displaces Bax from binding the anti-apoptotic Bcl-2 and Bcl-X<sub>L</sub> proteins, leading to outer membrane permeability and apoptosis [206]. Consistent with a role for Akt signaling in MEF survival following oxidative stress, stimulation of wild-type MEFs with hydrogen peroxide resulted in Akt (Thr<sup>308</sup>/Ser<sup>473</sup>) activation and a dramatic increase in phosphorylated Bad (Ser<sup>136</sup>) (Fig. 3.6D). However, in Rit null cells, the phosphorylation of both Akt and Bad proteins was significantly reduced following hydrogen peroxide exposure. These data provide additional evidence that Rit loss selectively alters the coupling of oxidative stress to intracellular signaling pathways, particularly p38-dependent Akt activation.



**Figure 3.6 Rit Contributes to p38-MK2-HSP27-Akt Pro-Survival Signaling.** (A) Wild-type and Rit<sup>-/-</sup> MEFs were serum starved for 3 h prior to the stimulation of H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M for indicated times). The total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. (B) The relative phosphorylation of MK2, HSP27 and Akt after 15 min H<sub>2</sub>O<sub>2</sub> exposure was quantified and compared between wild-type (black bars) and Rit<sup>-/-</sup> (white bars) MEFs. The results are presented as mean  $\pm$  S.D. (*t*-test; \*  $p < 0.05$ , \*\*  $p < 0.01$ ,  $n \geq 4$ ). (C) Wild-type and Rit<sup>-/-</sup> MEFs were serum starved for 3 h prior to the stimulation of EGF (100 ng/ml for indicated times). The total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. (D) Wild-type and Rit<sup>-/-</sup> MEFs were serum starved for 3 h prior to the stimulation of H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M for indicated times). The total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies.

To test the above hypothesis, wild-type MEFs were treated with SB203580 (10  $\mu$ M) prior to hydrogen peroxide exposure, reasoning that p38 blockade should disrupt Akt phosphorylation and increase oxidative stress-dependent apoptosis. As shown in Figure 3.7A, p38 inhibition, but not MEK/ERK blockade, resulted in a dramatic reduction in cell viability, rivaling that seen in *Rit*<sup>-/-</sup> MEFs, following hydrogen peroxide exposure. As expected, p38 inhibition was found to disrupt hydrogen peroxide-mediated MK2 and HSP27 phosphorylation in wild-type MEFs (Fig. 3.7B). As a control, we also examined the effect of p38 blockade on EGF-mediated signaling. While inhibition of p38 signaling blocked EGF-mediated MK2 and HSP27 phosphorylation, SB203580 treatment had no effect on EGF-dependent Akt signaling (Fig. 3.7C), but did suppress Akt activity following hydrogen peroxide exposure (Fig. 3.7B). These results support a critical role for p38-dependent Akt activation in the survival of fibroblasts responding to oxidative stress.



**Figure 3.7 p38 Signaling Promotes Akt in Response to ROS.** (A) Wild-type or *Rit*<sup>-/-</sup> MEFs were treated with H<sub>2</sub>O<sub>2</sub> (150  $\mu$ M) for 12 or 24 h in the presence or absence of the p38 inhibitor SB203580 (SB, 10  $\mu$ M) or MEK inhibitor PD98059 (PD, 10  $\mu$ M). Cell viability was determined by MTS metabolism at indicated times after initial exposure of H<sub>2</sub>O<sub>2</sub>. The results are presented as mean  $\pm$  S.D. (*t*-test; \*\* *p* < 0.01, *n* = 3). (B) Wild-type MEFs were serum starved for 3 h and then pre-treated with SB203580 (10  $\mu$ M) for 30 min prior to the stimulation of H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M for indicated times). The total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. (C) Wild-type MEFs were serum starved for 3 h and then pre-treated with SB203580 (10  $\mu$ M) for 30 min prior to the stimulation of EGF (100 ng/ml for indicated times). The total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies.

## DISCUSSION

Reactive oxygen species participate in a diverse array of biological processes, including cell growth, cellular senescence and apoptosis [190, 193, 207]. In large part, this diversity of cellular processes is due to ROS-mediated activation of canonical signaling pathways, including multiple MAPK cascades [190, 193]. This chapter describes an unexpected role of Rit GTPase in cell survival in response to ROS stress. p38 MAPK appears a major target of Rit in response to oxidative stress and represents a novel survival mechanism, in which p38 MAPK modulates Akt activity through p38-MK2-HSP27 signaling complex.

The creation of mice lacking expression of endogenous Rit GTPase provided a system to define the essential physiological function of Rit protein. While previous studies using ectopic expression of active or dominant negative mutants of Rit or RNAi-based gene silencing have identified a role of Rit in the regulation of neuronal differentiation and morphogenesis [109, 111] and for Rit and RIC in differentiation [93, 95, 99, 107], the current genetic model establish Rit as a crucial regulator of a pro-survival cascade in response to oxidative stress, which cannot be compensated by other Ras family G-proteins. Together with our previous data showing *Drosophila* lacking *dRIC*, the ortholog of Rit, display higher susceptibility to environmental stresses than wild-type flies [183], Rit appears to have the conserved ability to regulate cell survival in a p38-dependent fashion.

HSP27 is well known as a pro-survival molecule. Overexpression of HSP27 alone is sufficient to promote cell survival in response to diverse cellular stresses in numerous studies [74, 208, 209]. Several studies also demonstrated that phosphorylated HSP27 displays anti-apoptotic activity [210, 211]. The exact mechanism, however, is yet fully understood. Recent studies from several groups have described a novel signaling module, in which HSP27 serves as a scaffold to couple p38/MK2 signaling to Akt-dependent survival [127, 200-202]. Our data indicate that Rit functions as a critical upstream regulator of HSP27 phosphorylation in response to oxidative stress, and in the control of p38/MK2/HSP27/Akt signaling [112]. However, it is still unclear whether the contribution of HSP27 to Rit-mediated pro-survival effect is mediated primarily by coordinating ROS-dependent Akt activation or whether phosphorylated HSP27 supports

additional distinct survival mechanisms. Further studies are needed to address this question.

Hydrogen peroxide has the capacity to function as a proliferative signal [212], a second messenger [213], or as a signal to promote apoptosis or cell survival [214, 215]. Hydrogen peroxide exposure promotes activation of all the classic MAPK cascades [216], and it appears that cell fate is dictated by the interplay of these individual kinase pathways [196-198]. Our data suggest that Rit contributes to this process by directing p38-dependent cell survival. How might Rit achieve this effect? Scaffolding proteins are known to confer specificity, promoting distinct spatial and temporal control over p38 activation and directing coupling to specific downstream effector pathways [217]. The coordinated loss of hydrogen peroxide-mediated, but not EGF-dependent, p38, MK2, HSP27, and Akt activation, suggests that Rit plays a critical role in coordinating p38-mediated Akt activation and survival. A large number of regulatory kinases are known to contribute to p38 activation including MAPK kinase (MAPKK)-dependent and MAPKK-independent pathways [195, 197]. An important goal of future studies is to determine the molecular nature of the coupling between Rit and p38. Just as the recently identified OSM scaffold is known to specify Rac GTPase-mediated p38 activation following hyperosmotic stress by generating a Rac-OSM-MEKK3-MKK3 regulatory module [62], our data suggest a model in which Rit functions as an upstream regulator of a scaffolded p38-MK2-HSP27 complex involved in ROS-dependent Akt activation. Studies are ongoing to test this model.

Identifying a role for a Ras GTPase in Akt-mediated cell survival is perhaps not surprising. A number of Ras subfamily GTPases are known to direct PI3-kinase-dependent Akt activation [218, 219], with Akt in turn controlling the balance between survival and apoptosis [220]. Indeed, we find that oxidative stress activates Akt, resulting in Bad phosphorylation (Fig. 3.6D), and that both Rit and p38 contribute to this signaling cascade (Figs. 3.6 and 3.7). Akt is activated by two sequential phosphorylation events, with PDK1 directing Thr<sup>308</sup> phosphorylation, and a less well defined “PDK2” activity directing Ser<sup>473</sup> phosphorylation [117]. A complex interplay has been described for Akt, HSP27, p38 and MK2 kinases, in which p38/MK2 signaling induces both Akt Ser<sup>473</sup> phosphorylation and the release of activated Akt from phosphorylated HSP27, although



the molecular mechanisms regulating the signaling and dynamics of the HSP27 scaffolded complex remain to be established [127, 200-202]. Therefore, while activation of Akt is known to underlie the ability of a number of Ras GTPases to promote cell survival, Rit appears to control a distinct p38-mediated survival signaling cascade, which relies in part upon ROS-dependent p38-MK2-HSP27-Akt activation. Studies are underway to determine whether Rit uniquely contributes to p38/MK2-Akt signaling, which might explain why other Ras family GTPases cannot compensate for Rit loss. Stress-mediated p38 activation can lead to either cell death or cell recovery, depending on both the severity of the stimulus but also the nature of the cell [197]. It will be important to examine the contribution of Rit to cell survival in additional cell systems, especially, neurons, cardiomyocytes, and granulocytes, in which p38 signaling has been shown to support survival [221].

In summary, the data presented in this chapter demonstrate that the Rit GTPase is a key participant in oxidative stress signal propagation through the regulation of a p38-Akt cascade. The evolutionary conservation of this stress signaling pathway between mammals and *Drosophila* indicates its importance to cell survival, but potentially also for the regulation of additional p38-mediated biological processes [197] such as the regulation of cell cycle progression [221] and the lifespan of hematopoietic stem cells in response to oxidative stress [222]. p38 signaling has also been implicated in the regulation of additional pro-survival transcriptional cascades, including the activation of MEF2 [223],  $\beta$ -catenin via GSK3 inactivation [224], and MSK1/2-CREB cascades [225], expanding the potential role for Rit in regulating whether ROS-dependent p38 activity results in cell death or recovery.

## CHAPTER FOUR

### Rit-Mediated Akt<sup>Ser473</sup> Phosphorylation Requires mTORC2 Complex

#### INTRODUCTION

The serine/threonine protein kinase Akt/PKB is a critical regulatory kinase, controlling diverse cellular processes, including cell proliferation, differentiation, migration, nutrient metabolism, and programmed cell death (apoptosis) [114]. Of the diverse functions, one of most studied is the role for Akt in survival signaling. Akt controls multiple anti-apoptotic pathways in response to not only growth factors, but also to a variety of cellular stresses, including oxidative stress [114]. Upon activation, Akt acts by directly phosphorylating a variety of downstream substrates (such as Bad and caspase-9), but also regulating the activity of a collection of downstream kinases (such as mTOR, GSK3 $\beta$ ), which in turn regulate additional cellular targets such as transcription factors [77, 115-120]. As a result, Akt is able to regulate pro-survival signaling by controlling the activity of individual proteins but also by modulating gene transcription.

The activity of Akt is controlled by two distinct phosphorylation events. Phosphorylation at Thr<sup>308</sup> in the activation loop is controlled by the PDK1 kinase pathway and is essential for kinase activity [117]. However, maximal Akt activity requires a second phosphorylation event at Ser<sup>473</sup> within the C-terminal hydrophobic motif mediated by a less well defined “PDK2-like” pathway [121]. To date, multiple PDK2-like pathways have been reported, of which the mammalian TOR complex 2 (mTORC2) is the best characterized [122]. Studies from our group and others have suggested that the p38-MK2-HSP27 signaling complex functions upstream to control Akt Ser<sup>473</sup> phosphorylation, and it has been suggested that both p38 and/or MK2 may function as PDK-2 kinases in response to oxidative stress exposure [112, 127, 200-202]. In addition, DNA protein kinase (DNA-PK) has been reported to directly mediate Akt Ser<sup>473</sup> phosphorylation at the double strand break site in the nucleus following irradiation damage [128], leading the suggestion that potentially multiple PDK2-like kinase cascades serve to fine-tune Akt activity in response to distinct cellular stimuli.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth, proliferation, survival and motility [226-228]. mTOR is the

catalytic subunit of two evolutionarily conserved protein complexes: mTORC1 and mTORC2, which are distinguished from one another by their distinct subunit composition. mTORC1 is composed of mTOR, Deptor, mLST8 and the TORC1 specific subunits Raptor [229]. The immunosuppressant drug rapamycin binds to FK506-binding protein (FKBP12) after entering the cell and the complex inhibits mTORC1 activity by binding to Raptor [230]. The mTORC2 kinase complex contains mTOR, Deptor, mLST8 and the TORC2 specific subunits Rictor, Sin1 and Protor [229]. Due to the absence of the Raptor subunit, the mTORC2 complex is generally considered to be rapamycin resistant, although long-term treatment with rapamycin has been reported to inhibit mTORC2 activity [125]. The distinct composition of these complexes may explain their different patterns of activity, regulation, and distinct substrate specificity. mTORC1 is known to be regulated by growth factors, nutrient availability and the energy state of the cell, and to directly phosphorylate S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1) to regulate protein synthesis as well as other biological processes including autophagy [228]. mTORC2 is less well characterized. Recently, mTORC2 has been reported to phosphorylate AGC kinase family members Akt, PKC and SGK within a C-terminal hydrophobic motif to regulate cell survival, cell cycle progression, cytoskeleton rearrangement and motility [226]. However, our knowledge about the upstream regulation of mTORC2 is limited. PI3K and the subsequent substrate PI(3,4,5)P<sub>3</sub> have been shown to positively regulate mTORC2 activity, although PI3K-independent routes of mTORC2 regulation have also been reported [231, 232]. These data suggest that mTORC2 may function as a central hub to integrate diverse upstream signals from distinct pathways and to direct these diverse stimuli to a range of cellular processes.

Several Ras-related small GTPases have also been shown to be required for various aspects of mTORC2 activity. For instance, genetic studies revealed Ryh1 GTPase, a human Rab6 ortholog in fission yeast, controls the TORC2 activity to mediate vacuolar integrity and cellular stress resistance in yeast [233]. RasC, a human H-Ras ortholog in *Dictyostelium*, contributes to TORC2-Akt pathway to regulate F-actin dynamics and *Dictyostelium* chemotaxis [133]. Recently, in mammalian cells, Rac1 has been shown to regulate both mTORC1 and mTORC2 subcellular localization by direct interaction with mTOR, thus controlling cell growth [227]. In addition, Rheb and Rag

family GTPases have recently been identified to be essential for the activation of mTORC1 [228, 234, 235]. Their role in the regulation of mTORC2 pathway, however, remains to be determined.

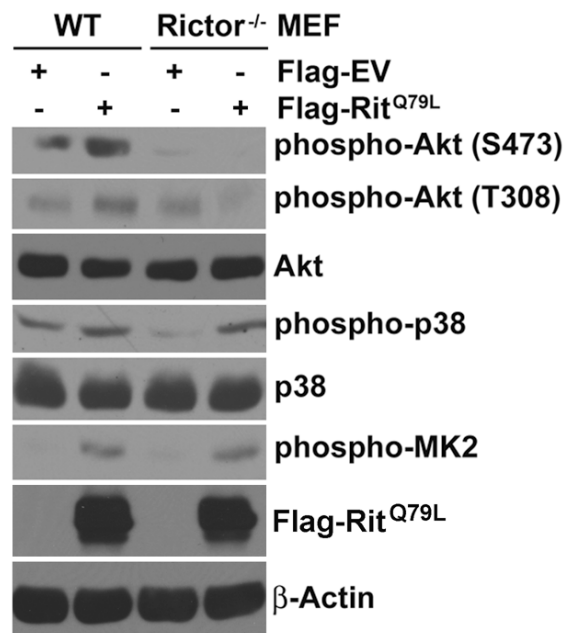
The data from previous chapter, along with our published studies [112], have shown that Rit controls the p38-MK2-HSP27 pathway, which in turn directs the phosphorylation of Akt at Ser<sup>473</sup> residue following oxidative stress. These results led us to ask whether the mTORC2 complex was operating downstream of Rit to control stress-dependent Akt activation. Here, using both Rictor null MEF and shRNA to knockdown mTORC2, we show that mTORC2 is required for constitutively active Rit- and H<sub>2</sub>O<sub>2</sub>-induced Akt phosphorylation at Ser<sup>473</sup>. mTORC2 kinase activity is enhanced by H<sub>2</sub>O<sub>2</sub> exposure, which may dependent upon the direct interaction of active Rit and Sin1, a specific subunit of the mTORC2 complex. Moreover, p38 MAPK appears key to ROS-dependent stimulation of mTORC2 kinase activity. Collectively, these data suggest that Rit coordinates p38-MK2-HSP27 signaling with the mTORC2 complex to control Akt phosphorylation following oxidative stress.

## RESULTS

### **mTORC2 Is Required for Rit<sup>Q79L</sup>-Mediated Phosphorylation of Akt at Ser<sup>473</sup>**

Our previous studies have shown that Rit acts upstream of the p38-MK2-HSP27 signaling complex to regulate Akt Ser<sup>473</sup> phosphorylation in response to oxidative stress. While it was clear that p38/MK2 activity was required for Akt phosphorylation, it was unclear whether Akt Ser<sup>473</sup> was a direct or indirect target of these kinases. Since mTORC2 is known to function as the “PDK2” required for insulin-mediated Akt Ser<sup>473</sup> phosphorylation, we investigated whether mTORC2 was required for Rit-mediated Akt phosphorylation. As shown in Fig. 4.1, overexpression of a constitutively active Rit (Rit<sup>Q79L</sup>) mutant in wild-type MEFs enhances Akt Ser<sup>473</sup> phosphorylation. However, Rit<sup>Q79L</sup> expression fails to stimulate Akt Ser<sup>473</sup> phosphorylation in Rictor null MEFs, which do not express functional mTORC2. This data indicates that mTORC2 is essential for Rit-mediated Akt stimulation. Rit-mediated p38 MAPK and MK2 activation is normal in Rictor<sup>-/-</sup> MEFs, suggesting that mTORC2 either mediates Akt Ser<sup>473</sup> phosphorylation

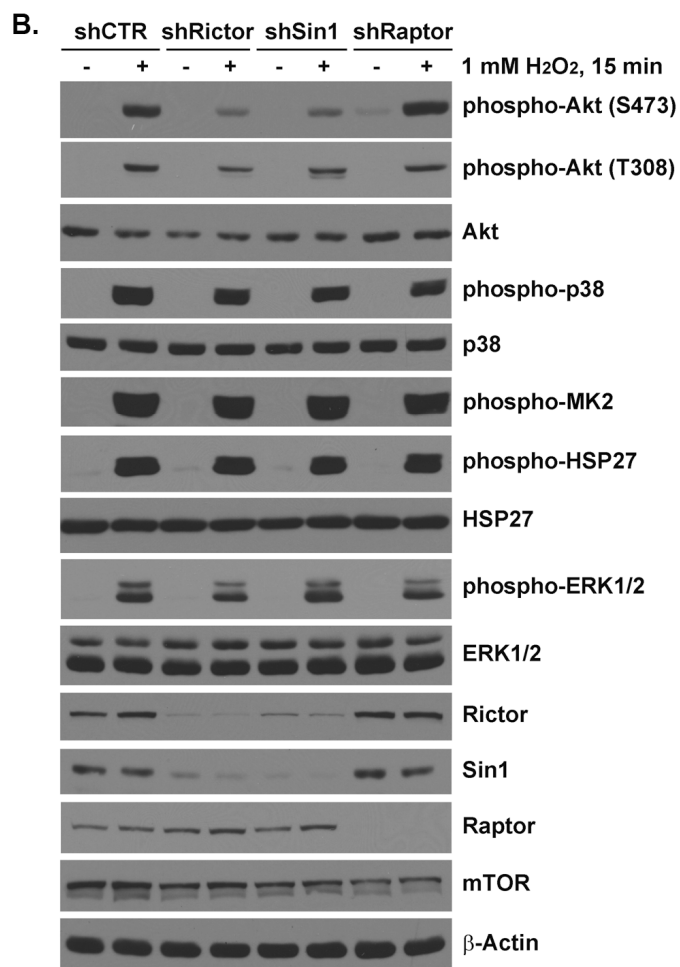
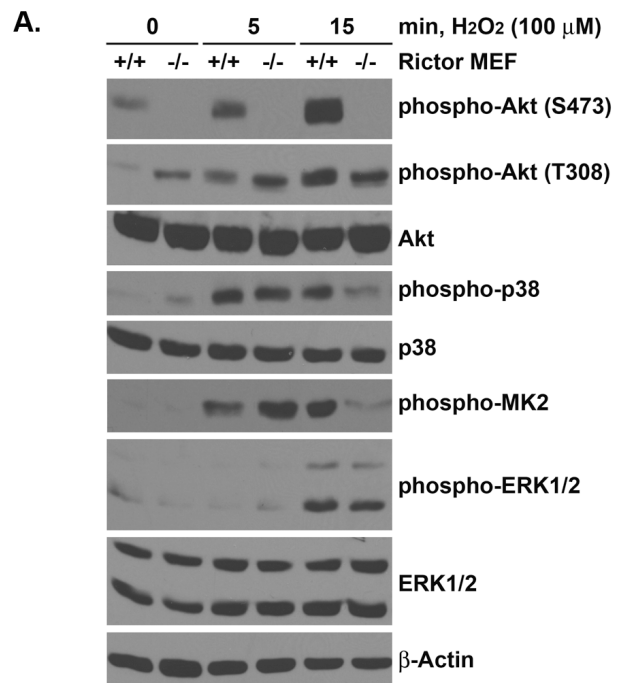
in a p38/MK2-independent fashion or that TORC2 operates downstream of p38/MK2 within this signaling cascade.



**Figure 4.1 mTORC2 Is Required for Rit<sup>Q79L</sup>-Mediated Phosphorylation of Akt Ser<sup>473</sup>.** Wild-type and Rictor<sup>-/-</sup> MEFs were transfected with 3×Flag-Rit<sup>Q79L</sup> or empty vector for 48 h. Cells were then subjected to serum starvation for 3 h prior to the preparation of total cell lysates. Cell lysates (40 µg) were analyzed by immunoblotting with the indicated antibodies.

### **H<sub>2</sub>O<sub>2</sub>-Induced Phosphorylation of Akt at Ser<sup>473</sup> Requires mTORC2**

Since Rit contributes to the induction of Akt phosphorylation at Ser<sup>473</sup> following H<sub>2</sub>O<sub>2</sub> exposure, we speculated that mTORC2 may be also required for ROS-mediated Akt activation. To address this question, wild-type and Rictor<sup>-/-</sup> MEFs were treated with H<sub>2</sub>O<sub>2</sub> (Fig. 4.2A). As expected, Akt Ser<sup>473</sup> phosphorylation was completely abolished in Rictor null MEFs following H<sub>2</sub>O<sub>2</sub> exposure, while PDK1-mediated Thr<sup>308</sup> phosphorylation, as expected, was unaffected. Moreover, the kinetics and amplitude of p38, MK2 and ERK activation remained intact in Rictor null MEFs. This was not an artifact arising from the use of Rictor<sup>-/-</sup> MEFs, since shRNA-mediated silencing of either endogenous Rictor or Sin1 in HeLa cells significantly reduced H<sub>2</sub>O<sub>2</sub>-mediated phospho-Akt (Ser<sup>473</sup>) levels without affecting the phosphorylation of p38, MK2, HSP27, or ERK (Fig. 4.2B). As expected, knocking-down Raptor expression, and thus disrupting the mTORC1 complex, had no significant effect on ROS-mediated Akt Ser<sup>473</sup> phosphorylation. Interestingly, phospho-Akt (Thr<sup>308</sup>) level is decreased following Rictor or Sin1 silencing. This is perhaps not surprising since it has been reported that Ser<sup>473</sup> phosphorylation serves to enhance and stabilize Thr<sup>308</sup> phosphorylation [236]. Taken together, these data indicate that mTORC2 is a key regulator of Akt Ser<sup>473</sup> phosphorylation following H<sub>2</sub>O<sub>2</sub> exposure.

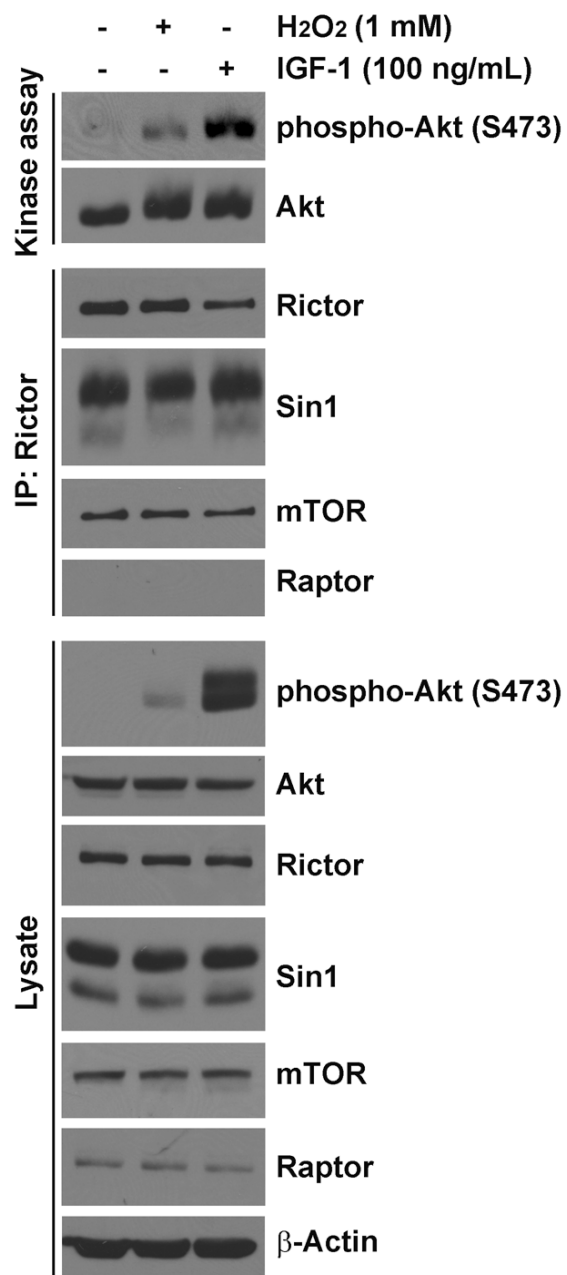




**Figure 4.2 H<sub>2</sub>O<sub>2</sub>-Induced Phosphorylation of Akt at Ser<sup>473</sup> Requires mTORC2.** (A) Wild-type and Rictor<sup>-/-</sup> MEFs were serum starved for 3 h prior to H<sub>2</sub>O<sub>2</sub> exposure (100  $\mu$ M for 15 min). Total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. (B) HeLa cells were exposed to recombinant lentivirus expressing shRNAs against Rictor, Sin1 or Raptor, and cultured under puromycin (2  $\mu$ g/mL) selection for 4 days to enrich for infected cells. The resultant cultures were serum starved for 4 h prior to H<sub>2</sub>O<sub>2</sub> exposure (1 mM, for 15 min). Total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. Note that the lentiviral encoded shRNAs efficiently attenuate Rictor, Sin1 and Raptor expression, and that Akt Ser<sup>473</sup> phosphorylation requires Rictor and Sin1, but not Raptor.

### **H<sub>2</sub>O<sub>2</sub> Enhances mTORC2 Kinase Activity**

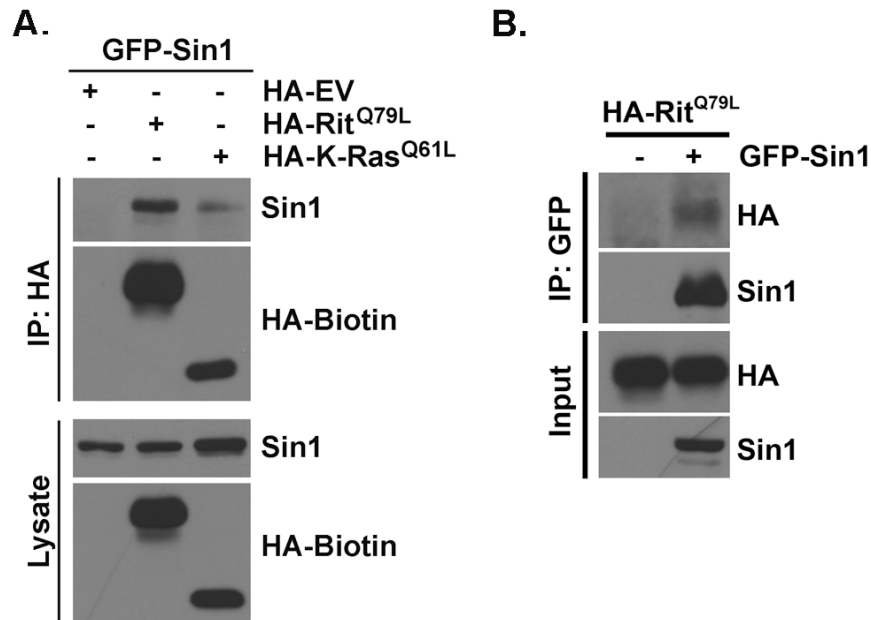
We next examined the kinase activity of mTORC2 following H<sub>2</sub>O<sub>2</sub> exposure. We immunoprecipitated the whole mTORC2 complex using the antibody against mTORC2 specific subunit Rictor, *in vitro* kinase analysis of anti-Rictor immunoprecipitates find that mTORC2 kinase is elevated following both H<sub>2</sub>O<sub>2</sub> and IGF-1 stimulation (Fig. 4.3, *upper panel*), mirroring the Ser<sup>473</sup> phosphorylation state of endogenous Akt from these same cell lysates (Fig. 4.3, *lower panel*). Thus, H<sub>2</sub>O<sub>2</sub> stimulates mTORC2 kinase activity, resulting in elevated phosphorylation of Akt within its hydrophobic motif.



**Figure 4.3 H<sub>2</sub>O<sub>2</sub> Stimulates mTORC2 Kinase Activity.** HeLa cells were serum starved for 4 h prior to H<sub>2</sub>O<sub>2</sub> exposure (1 mM for 15 min). Total cell lysates (1 mg) were prepared and subjected to anti-Rictor antibody immunoprecipitation. The immunoprecipitated fraction was subjected to mTORC2 *in vitro* kinase assay using recombinant His-tagged Akt (200 ng) as substrate. The immunoprecipitated fractions and total cell lysates were analyzed by immunoblotting with the indicated antibodies. In the *top panel* shows the phospho-Akt Ser<sup>473</sup> level as well as the total Akt in the kinase reaction. The *middle panel* shows the efficiency and specificity of mTORC2 purification. The *bottom panel* demonstrates equal loading and H<sub>2</sub>O<sub>2</sub>-mediated phosphorylation of endogenous Akt at Ser<sup>473</sup>. Note that the activation of endogenous phospho-Akt Ser<sup>473</sup> resembles that seen using the *in vitro* mTORC2 kinase assay.

### **Rit Associates with mTORC2 Specific Subunit Sin1**

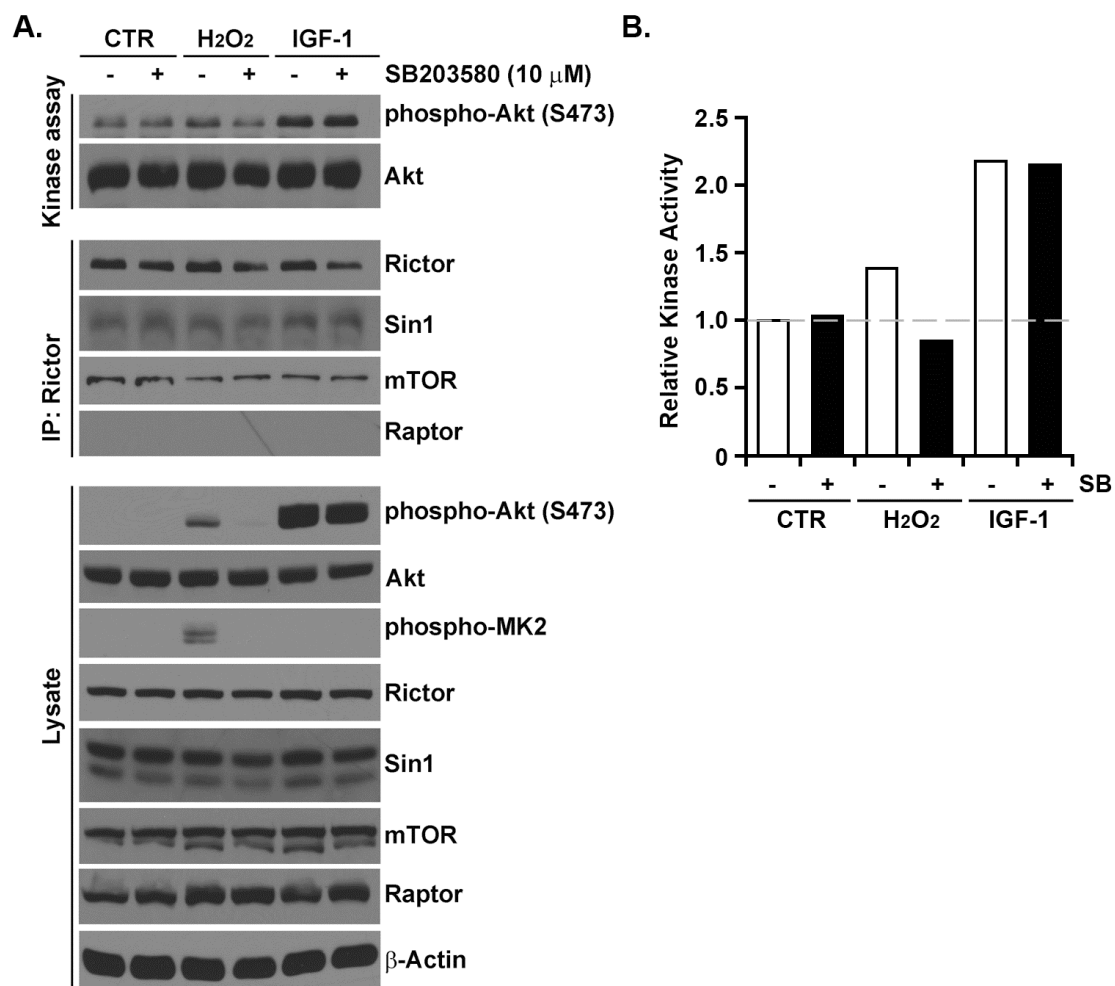
We next asked whether Rit was involved in ROS-mediated mTORC2 regulation. Sin1 contains a Raf-like Ras-binding domain (RBD), which has been shown to mediate the preferential interaction of Sin1 with active K-Ras over H-Ras [237]. This result prompted us to examine whether active Rit directly associates with Sin1. As seen in Figure 4.4A, anti-HA immunoprecipitates from HEK-293T co-transfected with GFP-tagged Sin1 and either active Rit (HA-Rit<sup>Q79L</sup>) or active K-Ras (HA-K-Ras<sup>Q61L</sup>) as a positive control, demonstrated Sin1 interaction with both K-Ras and Rit. The interaction with Rit GTPase was retained in a reverse immunoprecipitation, using anti-GFP antibodies to pull-down GFP-Sin1 (Fig. 4.4B). These data suggest that Rit may play a direct role in modulating mTORC2 activity.



**Figure 4.4 Active Rit Associates with the mTORC2 Specific Subunit Sin1.** (A) HEK-293T cells were co-transfected with HA-EV, HA-Rit<sup>Q79L</sup> or HA-K-Ras<sup>Q61L</sup> along with GFP-Sin1. 48 h after transfection, cells were serum starved for 12 h prior to cell lysis. Total cell lysates (500 µg) were subjected to immunoprecipitation with anti-HA antibody. The immune-complexes as well as the total cell lysates were analyzed by Sin1 and HA-Biotin immunoblotting. Note that both Rit and K-Ras are capable of interacting with Sin1. (B) HEK-293T cells were co-transfected with GFP-EV or GFP-Sin1 along with HA-Rit<sup>Q79L</sup>. 48 h after transfection, the cells were serum starved for 12 h prior to cell lysis. Total cell lysates (500 µg) were subjected to anti-GFP immunoprecipitation. The immune-complexes as well as total cell lysates were analyzed by Sin1 and HA immunoblotting. Note that this reverse IP also detects the interaction between active Rit and Sin1.

### **p38 MAPK Is an Upstream Regulator of mTORC2 in Response to Oxidative Stress**

We next asked whether p38 MAPK was required for ROS-mediated mTORC2 activity. As seen in Figure 4.5, p38 MAPK inhibition (10  $\mu$ M SB203580) was found to block mTORC2 kinase activity following H<sub>2</sub>O<sub>2</sub> treatment but not IGF-1, which mirrors the reduction seen in endogenous Akt Ser<sup>473</sup> phosphorylation. Taken together, these data strongly suggest that p38 MAPK acts as an upstream regulator of mTORC2 specifically in ROS signaling, with Rit/p38 signaling needed to couple ROS to mTORC2-mediated Akt activation.

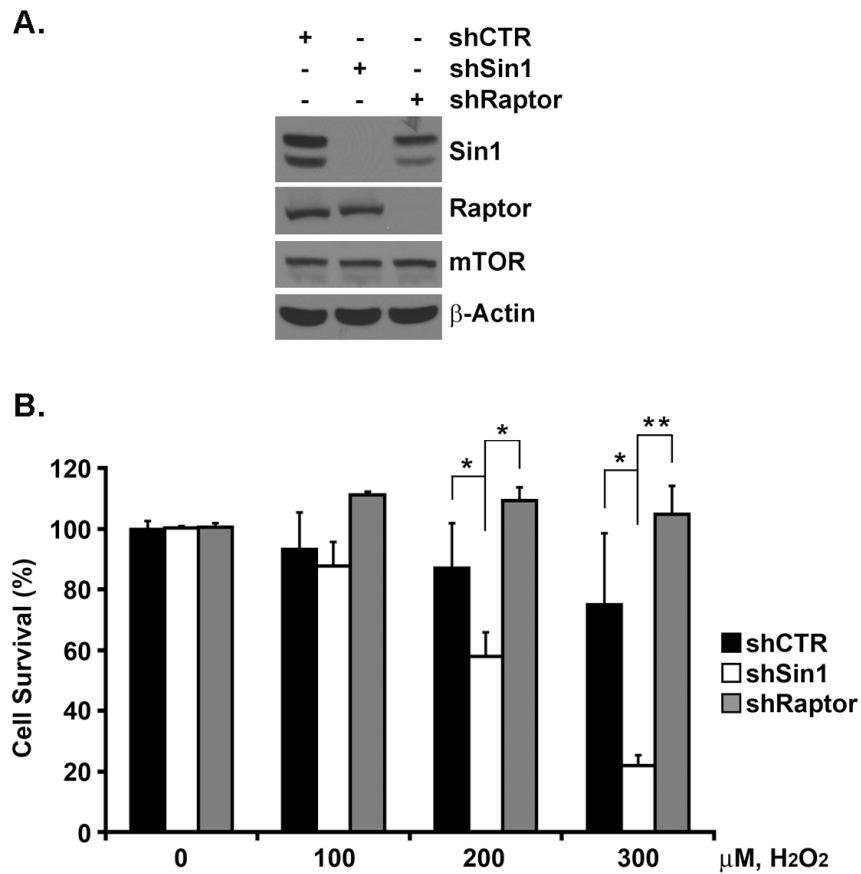




**Figure 4.5 p38 MAPK Controls mTORC2 Kinase Activity in Response to Oxidative Stress.** (A) HeLa cells were serum starved for 4 h followed by treatment with or without SB203580 (10  $\mu$ M) for 30 min prior to H<sub>2</sub>O<sub>2</sub> (1 mM for 15 min) and IGF-1 (100 ng/mL for 10 min) exposure as indicated. After cell lysis, total cell lysates (1 mg) were subjected to immunoprecipitation with anti-Rictor antibody, and the pelleted complex subjected to *in vitro* TORC2 kinase assay using His-tagged recombinant Akt (200 ng) as substrate. Fractions from the kinase reactions and total cell lysates were analyzed by immunoblotting with the indicated antibodies. In the *upper panel* shows the phospho-Akt Ser<sup>473</sup> level as well as the total Akt in the kinase reaction. The *middle panel* shows the efficiency and specificity of mTORC2 purification. The *lower panel* demonstrates equal loading and H<sub>2</sub>O<sub>2</sub>- and IGF-1-mediated phosphorylation of endogenous Akt at Ser<sup>473</sup>. Note the similar results seen using endogenous phospho-Akt Ser<sup>473</sup> immunoblotting and *in vitro* mTORC2 kinase analysis. (B) Quantification of mTORC2 kinase activity.

### **mTORC2 Deficiency Sensitizes Cells to Oxidative Stress**

mTORC2 has proven to be a central regulator of diverse biological processes including cell cycle regulation, cytoskeleton rearrangement, as well as the cellular stress response [226]. Since mTORC2 appears critical to H<sub>2</sub>O<sub>2</sub>-mediated Akt activation, we would expect mTORC2 deficient cells to be more vulnerable to oxidative stress. To evaluate cell survival after shRNA-mediated mTORC2 knockdown, HeLa cell cultures were left untreated or were exposed to increasing doses of H<sub>2</sub>O<sub>2</sub> for 12 h and viable cell numbers were determined using the MTS assay. shRNA expression was shown to efficiently knock down expression of both endogenous Sin1 and Raptor (Fig. 4.6A). Upon H<sub>2</sub>O<sub>2</sub> exposure, cell viability decreased in a dose-dependent manner. Importantly, attenuated expression of Sin1, required for mTORC2 function, dramatically exacerbated H<sub>2</sub>O<sub>2</sub>-induced cell death (200  $\mu$ M H<sub>2</sub>O<sub>2</sub>, 86.9%  $\pm$  14.9% and 58.0%  $\pm$  7.9% shCTR and shSin1, respectively,  $p < 0.05$ ) (Fig. 4.6B). Loss of endogenous mTORC1 following Raptor knockdown, however, did not result in increased sensitivity to oxidative stress, instead it was found to modestly promote cell survival (200  $\mu$ M H<sub>2</sub>O<sub>2</sub>, 86.9%  $\pm$  14.9% and 109.3%  $\pm$  4.3% shCTR and shRaptor, respectively,  $p > 0.1$ ) (Fig. 4.6B). Together, these data indicate that mTORC2 plays a critical role in promoting cell survival following oxidative stress.

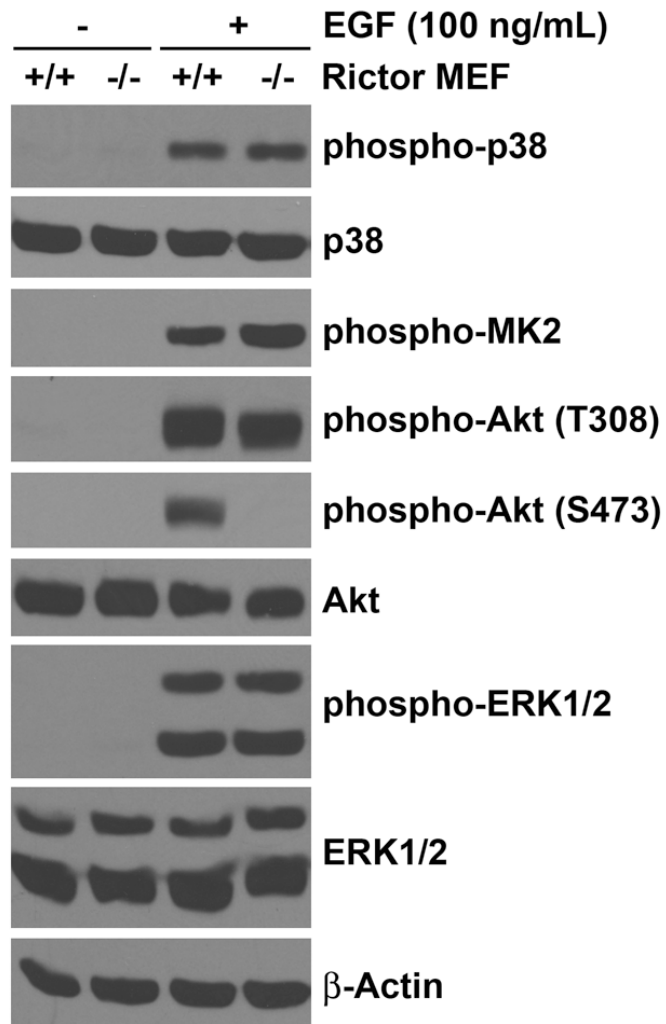


**Figure 4.6 mTORC2, but not mTORC1, Contributes to Cell Survival in Response to Oxidative Stress.** (A) HeLa cells were infected with lentivirus expressing shRNA against either Sin1 or Raptor, and cultured under puromycin selection (2  $\mu$ g/mL) for 4 days. Total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. Note that the lentiviral shRNA-mediated knock-down of Sin1 and Raptor is very efficient. (B) Following shRNA expression, HeLa cells (shCTR, shSin1 and shRaptor) were treated with the indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 12 h and cell viability determined by MTS assay. The results are presented as mean  $\pm$  S.D. (*t*-test; \*  $p < 0.05$ , \*\*  $p < 0.01$ ,  $n = 3$ )

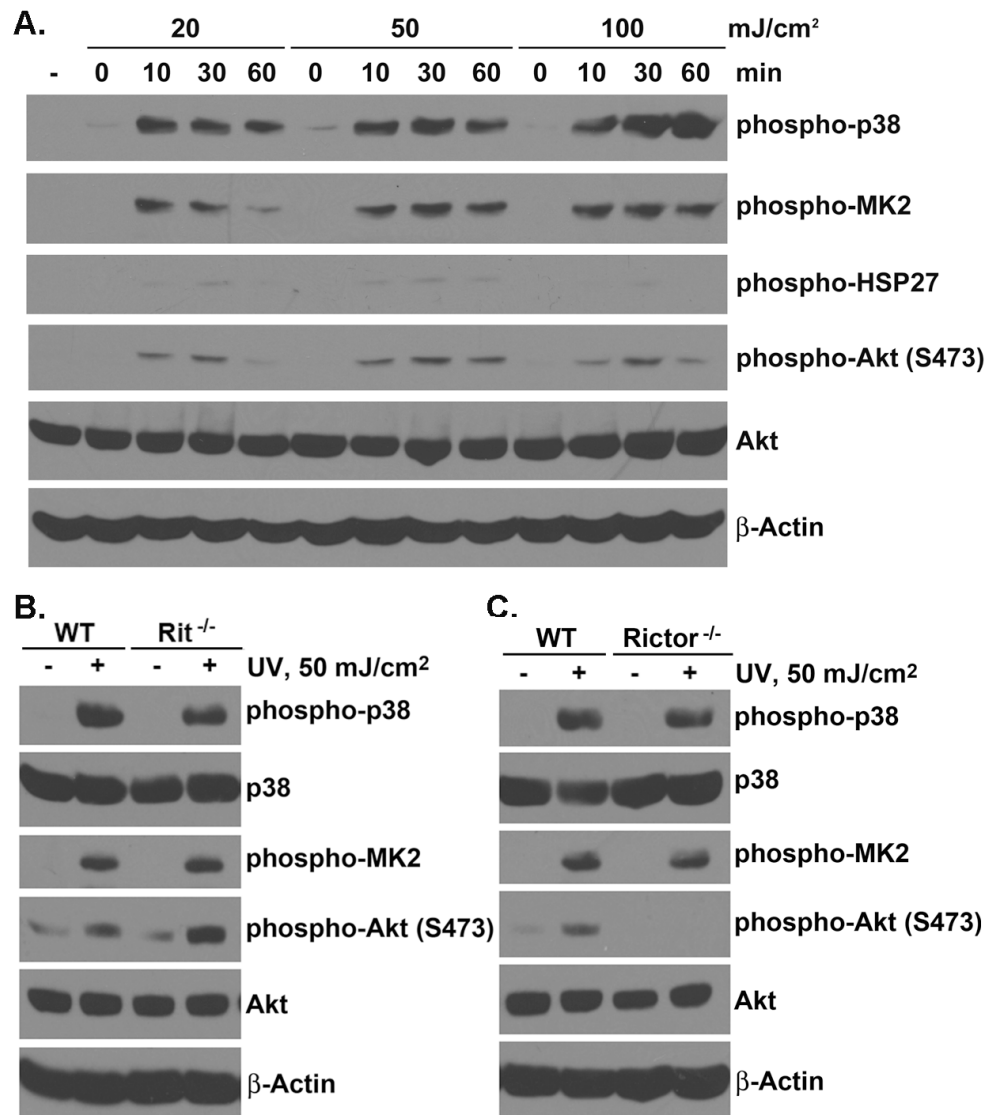
### **mTORC2 Functions as the PDK2 Pathway in Response to a Variety of Stimuli**

The requirement for TORC2 in ROS-dependent Akt activation, led us to next ask whether mTORC2 is required for other stimuli-induced Akt phosphorylation. In agreement with data indicating the importance of TORC2 to IGF-1/insulin-mediated Akt Ser<sup>473</sup> activation, loss of Rictor completely abolished Ser<sup>473</sup> phosphorylation of Akt without affecting Thr<sup>308</sup> phosphorylation of Akt following EGF stimulation. Since Rit is not essential in EGF-mediated Akt phosphorylation (Fig. 3.6C), mTORC2 appears to regulate both Rit-dependent and Rit-independent Akt signaling cascades.

The importance of TORC2 to Akt Ser<sup>473</sup> regulation was further supported by the UV irradiation studies. UVA/UVB exposure is known to cause multiple cellular stresses including DNA damage. The phosphorylation of p38 MAPK, MK2, HSP27 as well as Akt (Ser<sup>473</sup>) was dramatically upregulated in wild-type MEFs following UVA/UVB exposure (Fig. 4.8A). Rit<sup>-/-</sup> MEFs display normal activation of p38, MK2 and Akt (Ser<sup>473</sup>) following UV exposure (Fig. 4.8B), suggesting that UV-induced Akt (Ser<sup>473</sup>) phosphorylation is independent of Rit signaling. However, Akt Ser<sup>473</sup> phosphorylation was completely abolished in Rictor<sup>-/-</sup> MEFs (Fig. 4.8C). Taken together, these data support a critical role for mTORC2 downstream of a wide variety of extra- and intra-cellular stimuli in the control of Akt Ser<sup>473</sup> phosphorylation.



**Figure 4.7 mTORC2 Is Required for EGF-Mediated Akt Phosphorylation at Ser<sup>473</sup>.** Wild-type and Rictor<sup>-/-</sup> MEFs were serum starved for 3 h prior to EGF stimulation (100 ng/mL for 10 min). Total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies.



**Figure 4.8 UV-Dependent Phosphorylation of Akt Ser<sup>473</sup> Requires Functional mTORC2.** (A) Wild-type MEFs were serum starved for 2 h prior to being exposed to the indicated doses of UV irradiation. MEFs were then incubated for varying periods in serum-free DMEM to allow for recovery. Total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. (B) Wild-type and Rit<sup>-/-</sup> MEFs were serum starved for 3 h followed by 50 mJ/cm<sup>2</sup> UV exposure, allowed to recover for 30 min, and total cell lysates prepared and subjected to immunoblotting with the indicated antibodies. (C) Wild-type and Rictor<sup>-/-</sup> MEFs were serum starved for 3 h followed by 50 mJ/cm<sup>2</sup> UV exposure, allowed to recover (30 min), and total cell lysates subjected to immunoblotting with the indicated antibodies. Note that Akt Ser<sup>473</sup> phosphorylation requires mTORC2 but not Rit.

## DISCUSSION

In the present chapter, we present data suggesting that Rit/p38 signaling acts as an upstream regulator of mTORC2 to control Akt-mediated pro-survival signaling following oxidative stress. Moreover, mTORC2 is also essential for directing Akt Ser<sup>473</sup> phosphorylation in response to a variety of cellular stimuli including growth factors (EGF) and cellular stresses (ROS, UV irradiation), serving to extend the notion coming from the insulin signaling field that mTORC2 is a genuine “PDK2” kinase, to suggest that mTORC2 is perhaps the only cellular PDK2 kinase, with the mTORC2 complex acting to integrate multiple upstream signaling inputs to regulate Akt Ser<sup>473</sup> phosphorylation.

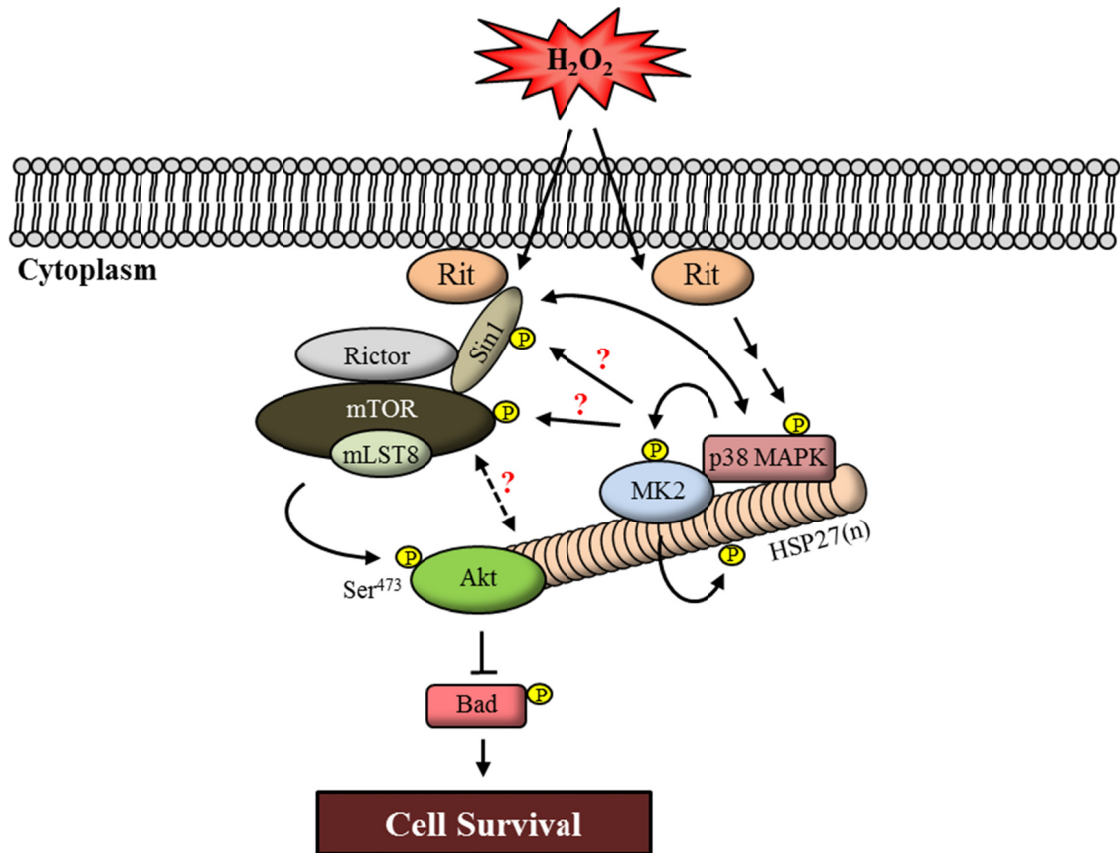
Akt activation is thought to require three steps: 1) membrane recruitment through the interaction of PH domain with PI (3,4,5) P<sub>3</sub> and PI (3,4) P<sub>2</sub> lipid head groups, which are produced by active PI3K; 2) phosphorylation of Thr<sup>308</sup> in the activation loop by PDK-1; and 3) phosphorylation of Ser<sup>473</sup> in the C-terminal hydrophobic motif via a less well defined “PDK-2” mechanism. Among the putative PDK-2-like kinases mTORC2 is the best characterized as an authentic regulator of Akt Ser<sup>473</sup> phosphorylation [114, 122]. The mechanism of mTORC2 regulation, however, remains elusive. Studies in this chapter provide evidence that both Rit and p38 MAPK are involved in mTORC2 activation in response to ROS.

Earlier reports have shown that the membrane localization of mTORC2 is essential for its biological activity in yeast [238]. The subcellular localization of mTORC2 is likely determined by Sin1, which contains a PH domain and a Raf-like Ras-binding domain, suggesting that Sin1, and by extension the entire mTORC2 complex, can be recruited to the plasma membrane in a PI3K- and/or small GTPase-dependent fashion [237, 238]. The interaction between active Rit and Sin1 suggests that mTORC2 may be dynamically recruited to the cell membranes in response to elevated levels of GTP-bound Rit. Since Rit is known to be activated by oxidative stress, this would provide a mechanism to couple ROS to mTORC2 activation.

It has been reported previously that both the p38-MK2-HSP27 cascade and mTORC2 complex act to regulate Akt phosphorylation at Ser<sup>473</sup> [112, 122, 127]. What is the relationship between these two complexes? Our data suggest that mTORC2 functions



downstream of the p38-MK2-HSP27 cascade since loss of mTORC2 completely abolished Akt Ser<sup>473</sup> phosphorylation without affecting p38, MK2 and HSP27 phosphorylation following oxidative stress exposure. How mTORC2 might be regulated downstream of Rit-p38 signaling remains an important research question (Fig. 4.9). Several lines of evidence support the formation of a larger scaffolded complex, in which mTORC2 would interact with one, or more, member of the Rit-p38-MK2-HSP27-Akt complex, bringing active mTORC2 in proximity to HSP27 bound Akt. Rit association may be sufficient to bridge these two complexes, since we demonstrate binding of Rit with Sin1 (Fig. 4.4), and previous studies have shown that active Rit is capable of interacting with the HSP27 complex [112]. In addition, earlier reports have documented a molecular interaction between Sin1 and p38, which is known to associate with the larger HSP27 scaffolded complex. Thus, it is possible that HSP27 bound p38 is involved in mTORC2 recruitment. Our earlier finding that HSP27 is required for Rit-mediated Akt activation supports this hypothesis [112], although an interaction between HSP27 and mTORC2 remains to be proven. Our studies, for the first time, identify a critical role for p38 MAPK signaling in mTORC2 activation. However, the underlying molecular mechanism remains to be determined. Studies from our lab and other groups suggest that p38/MK2 may serve as upstream kinases and mediate the phosphorylation of critical regulatory sites within the mTORC2 complex [239]. Indeed, previous studies have revealed that components of mTORC2 complex including mTOR, Rictor and Sin1 are hyper-phosphorylated upon stimulation. Phosphorylation at two serine sites is positively correlated with the mTOR kinase activity, with increased phosphorylation of Ser<sup>2448</sup> correlated with enhanced mTORC1 activity, and Ser<sup>2481</sup> phosphorylation linked to elevated mTORC2 activity [240]. But the kinase(s) responsible for directing these two phosphorylation events have yet been identified. It will be important to determine whether p38/MK2 controls mTORC2 complex via direct phosphorylation, and if so, to identify these p38/MK2-dependent phosphorylation sites.



**Figure 4.9 Proposed Model for Rit-p38-Mediated mTORC2-Dependent Akt Activation.**  $\text{H}_2\text{O}_2$  exposure stimulates the activation of Rit, which may simultaneously recruit mTORC2 and p38-MK2-HSP27-Akt signaling complexes to the plasma membrane. p38 MAPK is activated by Rit through a classical MAPKKK/MAPKK/MAPK cascade, although the putative MAPKKK/MAPKK involved in this pathway remain to be identified. Active p38 then phosphorylates MK2, which in turn phosphorylates HSP27. Meanwhile, p38 directly interacts with Sin1 and p38/MK2 may, therefore, phosphorylate mTORC2 on multiple sites to enhance the kinase activity of mTORC2 complex which serves as PDK2 to directly phosphorylate Akt at Ser<sup>473</sup>. HSP27 oligomers may also be required for the recruitment of mTORC2, bringing mTORC2 in close proximity to its substrate Akt. Finally, HSP27 phosphorylation by MK2 triggers the dissociation of this signaling complex and releases active Akt, which then promotes cell survival through multiple mechanisms including phosphorylating and inhibiting pro-apoptotic protein Bad.

mTORC2 has been shown to regulate a diverse array of physiological activities including cell metabolism, cell growth, and cytoskeleton rearrangement [226-228]. Here, we identify mTORC2 as an essential contributor to an Akt-dependent survival pathway in response to oxidative stress. However, the contribution of Rit signaling to mTORC2 activation suggests that Rit may contribute to the control of other mTORC2-dependent processes, including a role in the control of both SGK and PKC signaling [226]. One of the best characterized functions of SGK is to enhance the stability and expression of epithelial sodium channel to regulate ion transport [241]. PKC is known to play a critical role in neurite outgrowth for decades [242]. Therefore, it will be important to ask whether Rit/p38-mediated neurite outgrowth in PC6 cells as well as axonal/dendritic morphogenesis requires mTORC2-PKC activity, and examine whether Rit might also contribute to SGK control.

In summary, data in this chapter shows that  $H_2O_2$  and active Rit mediate mTORC2-dependent Akt Ser<sup>473</sup> regulation, thus eliciting a novel pro-survival signaling cascade in response to oxidative damage. Rit appears capable of stimulating mTORC2 complex kinase activity, and future studies are needed to determine whether this involves a direct interaction with the mTORC2 complex and/or an indirect p38-dependent mechanism involving phosphorylation of one or more proteins within the complex. Additional studies will be necessary to explore the importance of Rit to mTORC2 regulation of Akt signaling and whether Rit-mTORC2 signaling contributes to the regulation of other AGC family kinases.

## **CHAPTER FIVE**

### **Rit Promotes Selective Immature Neuron Survival following Oxidative Stress and Traumatic Brain Injury**

#### **INTRODUCTION**

Adult neurogenesis within the hippocampal dentate gyrus (DG) results in the continuous generation of new granule neurons which ultimately integrate into the neural circuitry throughout adulthood [134]. In this process, neural stem cells residing in the subgranular zone (SGZ) proliferate and differentiate into committed neural progenitor cells, i.e. doublecortin (Dcx) positive immature neurons, but also result in the production of astrocytes and oligodendrocytes [137]. A major fraction of these young neurons undergo apoptosis at early stages of development whereby birth, survival, and differentiation are influenced by countless endogenous and exogenous factors [154, 155]. Some factors like age, stress, and elevated glucocorticoid levels down regulate the formation of new nerve cells while others like gonadal hormones, several growth factors, or environmental stimulation promote increased neuron numbers [151, 152, 155, 163, 243-245]. The impact of factors associated with behavior and cognition suggest that the dynamics of adult neurogenesis is also regulated by the activity of existing hippocampal networks, although how this is conveyed to the progenitor cell population is still unclear. Surviving newborn immature neurons gradually migrate into the granular layer where they fully mature and incorporate into the hippocampal neural circuitry. This continuous production of granule neurons appears critical for maintenance of learning and memory, especially the storage of new spatial memories [135, 142, 143]. Disruption of normal adult neurogenesis by neurodegenerative diseases or brain injury has been linked to cognitive dysfunction in mouse models [144, 171-173], and thus there is great interest in understanding the control of neurogenesis, with the promise that modulation of this process might provide novel treatment strategies for a variety of neurodegenerative disorders.

Traumatic Brain Injury (TBI) is one of the major causes of death and disability worldwide, especially in children, athletes, and military personnel [246]. In addition to the physical damage resulting from the initial impact, brain trauma leads to secondary

injuries minutes, days, and even weeks following the initial trauma. These secondary injuries are triggered by a combination of events including excessive release of excitatory amino acids, accumulation of reactive oxygen species (ROS), altered calcium homeostasis, and excessive inflammatory responses [247]. As a result, patients suffer further neuron loss accompanied by the appearance of post-traumatic symptoms including cognitive deficits, depression, anxiety disorders, and seizures [248].

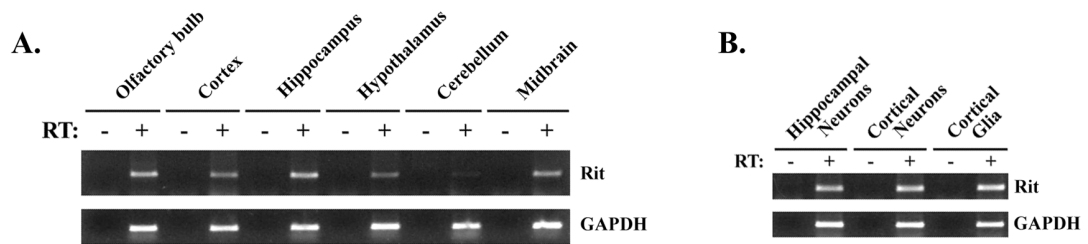
The hippocampus is particularly vulnerable to secondary injury following TBI, with both neural stem cells and new born immature neurons within the dentate gyrus (DG) undergoing high levels of apoptosis, essentially setting back the process of adult neurogenesis [173, 175]. It is postulated that this massive loss of neural stem cells and newborn immature neurons following the traumatic insult contributes to the cognitive dysfunction seen following head injury [181, 182]. Therefore, there is a great deal of clinical interest in understanding the molecular mechanisms underlying these events, especially in identifying neuroprotective mechanisms for these vulnerable neuron populations which might provide the basis for novel therapeutic strategies.

As demonstrated in the previous chapter and our recent publications, Rit mediates an evolutionarily conserved p38 MAPK-dependent pro-survival pathway in response to ROS [112, 183]. These studies have revealed that upon hydrogen peroxide exposure Rit controls a p38 MAPK-MK2-HSP27-Akt signaling cascade, serving to modulate Akt activity and its downstream targets including Bad. Since elevated ROS levels are one of the major causes of neuron loss following TBI [247], we speculated that Rit signaling may also play a key role in oxidative stress resistance in neurons following TBI. In this chapter, we confirm a critical role of Rit in p38-Akt signaling in primary hippocampal neurons in response to oxidative stress. Loss of endogenous Rit sensitizes primary hippocampal neurons, specifically Dcx-positive (Dcx<sup>+</sup>) newborn immature neurons to hydrogen peroxide. More importantly, *in vivo* studies indicate that Rit selectively promotes Dcx<sup>+</sup> newborn immature neuron survival in the DG following controlled cortical impact (CCI), a well-established experimental model of TBI. Thus, these studies are the first to identify a key role for a small GTPase in adult hippocampal neurogenesis, particularly a role for Rit directed intracellular signaling pathway(s) in the survival and ultimate integration of newborn neurons into the dentate gyrus.

## RESULTS

### Rit Is Widely Expressed in the Brain

Studies in primary mouse embryonic fibroblasts indicate that signaling by the small GTPase Rit functions to protect cells from oxidative stress (Chapter Three). To begin to determine whether Rit signaling plays a similar role in neurons, we first examined its expression in mouse brain. While we lack the necessary high affinity anti-Rit antibody to detect endogenous Rit protein, reverse transcriptase PCR (RT-PCR) analysis suggests that Rit is widely expressed in multiple brain subregions (Fig. 5.1A). In particular, the hippocampus appears to have high levels of Rit mRNA, consistent with *in situ* hybridization studies carried out by the Allen Brain Atlas (Gene name: Ras-like without CAAX 1; Image series: 69059924; Allen Institute for Brain Science, <http://www.brain-map.org/>). Brain tissue is composed primarily of two cell types, neurons and glial cells which can be further subdivided into astrocytes, microglia and oligodendrocytes. To distinguish between these general cell types, primary hippocampal neural and glial cultures were prepared from newborn mouse pups (P1, Post-natal day 1), and RT-PCR analysis used to indicate that both hippocampal neurons and glial cells express Rit (Fig. 5.1B). Collectively these data indicated that Rit is widely expressed throughout the brain.

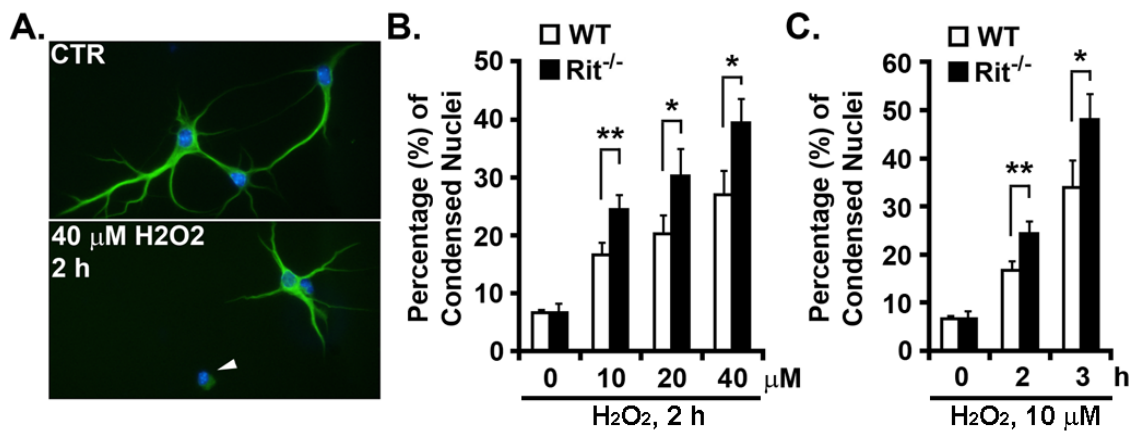


**Figure 5.1 Rit Protein Is Ubiquitously Expressed in the Mouse Brain.** (A) Total RNA was isolated from the indicated sub-regions of the C57BL6 mouse pup (P1) brain. Total RNA (2  $\mu$ g) was subjected to the RT-PCR with Rit selective primers using first strand cDNA as template. GAPDH served as control. (B) Total RNA was isolated from primary cultured hippocampal neurons, cortical neurons, and a mixed cortical glial cell population (DIV8). The presence of Rit mRNA was determined by RT-PCR analysis as described above. GAPDH was used as control.

### **Loss of Endogenous Rit Sensitizes Primary Hippocampal Neurons following Oxidative Stress**

Previous studies found that MEFs derived from Rit null mice are more vulnerable to oxidative stress. We wondered if Rit-mediated pro-survival signaling is preserved in neurons, a cell type known to be extremely sensitive to oxidative stress. To test this notion, hippocampal neuron cultures were isolated from Rit<sup>-/-</sup> P1 pups and the wild-type littermates. After 8 days *in vitro* (DIV8), neurons were left untreated or subjected to H<sub>2</sub>O<sub>2</sub> exposure, and apoptosis monitored by co-staining with anti-MAP2 (to identify neurons) and Hoechst (Fig. 5.2A). Despite displaying an equivalent percentage of apoptotic cells at base line (without H<sub>2</sub>O<sub>2</sub> exposure), Rit<sup>-/-</sup> hippocampal neurons had significantly higher levels of apoptosis following H<sub>2</sub>O<sub>2</sub> exposure. These increases in apoptosis were both dose and duration dependent (Fig. 5.2B, C), strongly suggesting that Rit supports oxidative stress survival signaling in neurons.

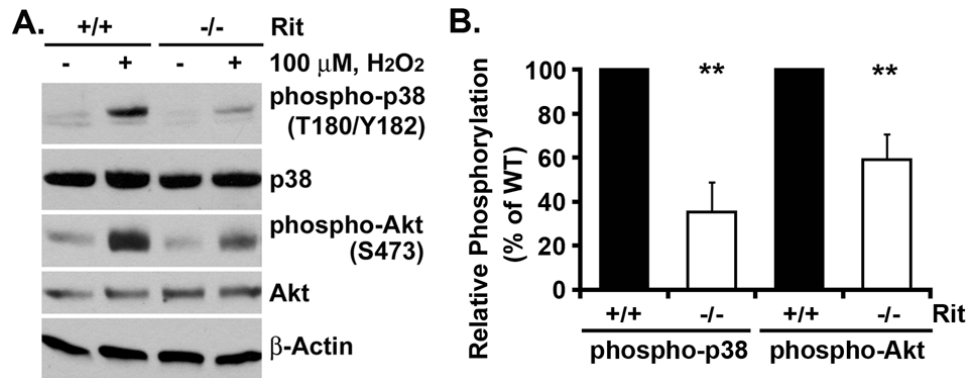




**Figure 5.2 Loss of Endogenous Rit Sensitizes Primary Hippocampal Neurons to Hydrogen Peroxide.** (A) Rit<sup>-/-</sup> and wild-type hippocampal neurons (DIV8) were left untreated or treated with the indicated dose of H<sub>2</sub>O<sub>2</sub> for the indicated duration, and immunostained with MAP2 antibody to label cell bodies and dendrites. Nuclei were visualized by Hoechst stain. *Panel A* shows representative images of Rit<sup>-/-</sup> neurons with or without H<sub>2</sub>O<sub>2</sub> treatment. An apoptotic neuron is highlighted by the arrowhead. (B, C) Percentage of neurons with condensed or fragmented nuclei following H<sub>2</sub>O<sub>2</sub> exposure. 10-15 fields were randomly selected for each treatment and scored for condensed nuclei. The data is presented as mean  $\pm$  S.D. (*t*-test: \*  $p < 0.05$ , \*\*  $p < 0.01$ ,  $n = 3$ )

### **Rit Loss Impairs p38 MAPK and Akt Phosphorylation in Response to ROS Exposure**

Recent studies have identified a novel p38-Akt signaling cascade downstream of Rit as playing a central role in oxidative stress survival. To determine whether a similar mechanism supports neural survival, wild-type and Rit<sup>-/-</sup> hippocampal neurons (DIV8) were starved in DMEM for 40 min followed by the exposure to H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M for 15 min). As expected, phosphorylation of p38 MAPK and Akt were significantly reduced in Rit null neurons (Fig. 5.3A). Compared with wild-type neurons, Rit<sup>-/-</sup> neurons had a 35.3%  $\pm$  13.6% (n = 5, p < 0.01) and 59.0%  $\pm$  11.6% (n = 3, p < 0.01) reduction in p38 and Akt phosphorylation, respectively (Fig. 5.3B). Taken together, these data suggest that Rit relies upon p38-Akt signaling in neurons to protect against oxidative stress.



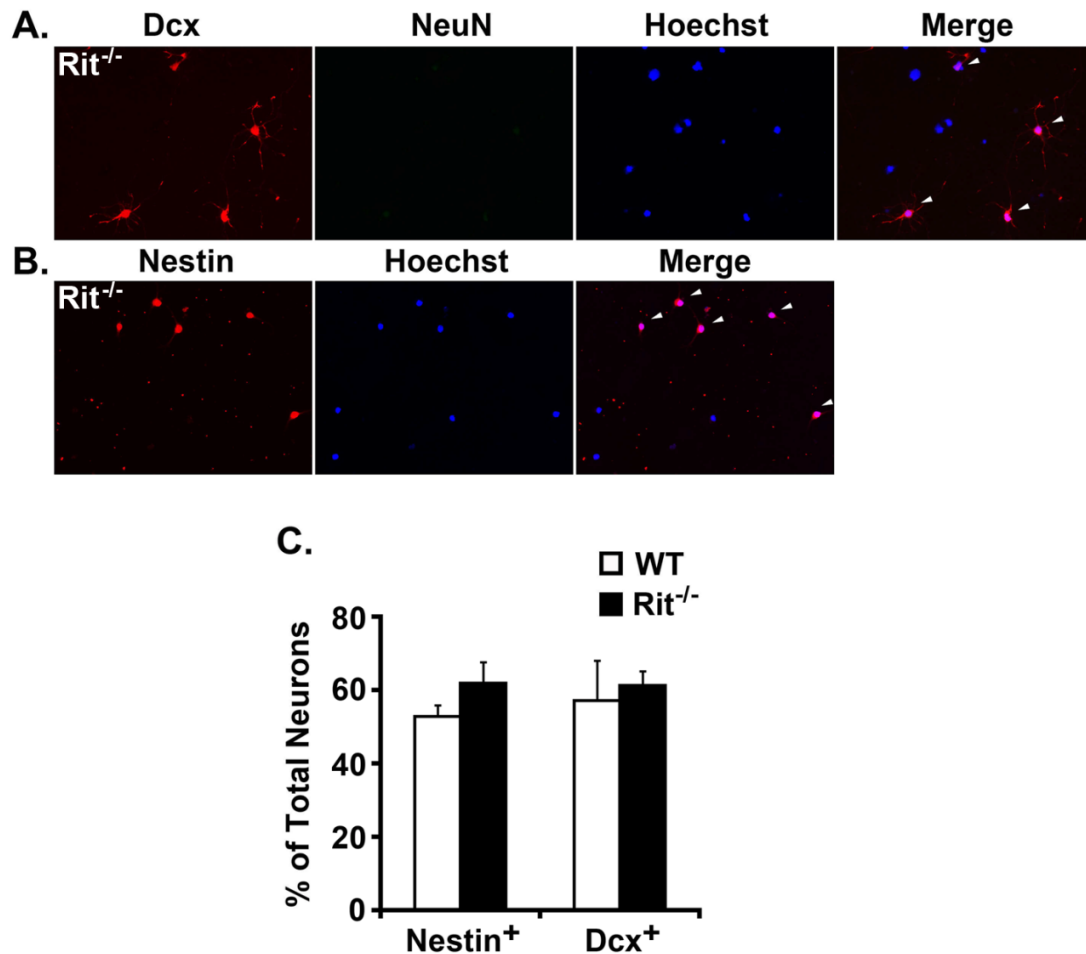
**Figure 5.3 Loss of Endogenous Rit Impairs ROS-Mediated p38 and Akt Activation.**

(A) Rit<sup>-/-</sup> and WT hippocampal neurons were cultured for 8 days (DIV8). On the day of the experiment, neurons were starved in serum-free DMEM for 40 min prior to H<sub>2</sub>O<sub>2</sub> exposure (100 μM for 15 min). Total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. Images are representative of five independent experiments. (B) Relative phosphorylation levels of p38 and Akt (Ser<sup>473</sup>) following H<sub>2</sub>O<sub>2</sub> exposure were determined by anti-phospho-specific immunoblotting. Band density was quantified by ImageJ. Note that both p38 and Akt phosphorylation levels are significantly reduced in Rit<sup>-/-</sup> neurons. The data is presented as mean ± S.D. (phospho-p38, n = 5, phospho-Akt, n = 3, t-test: \* p < 0.05, \*\* p < 0.01)

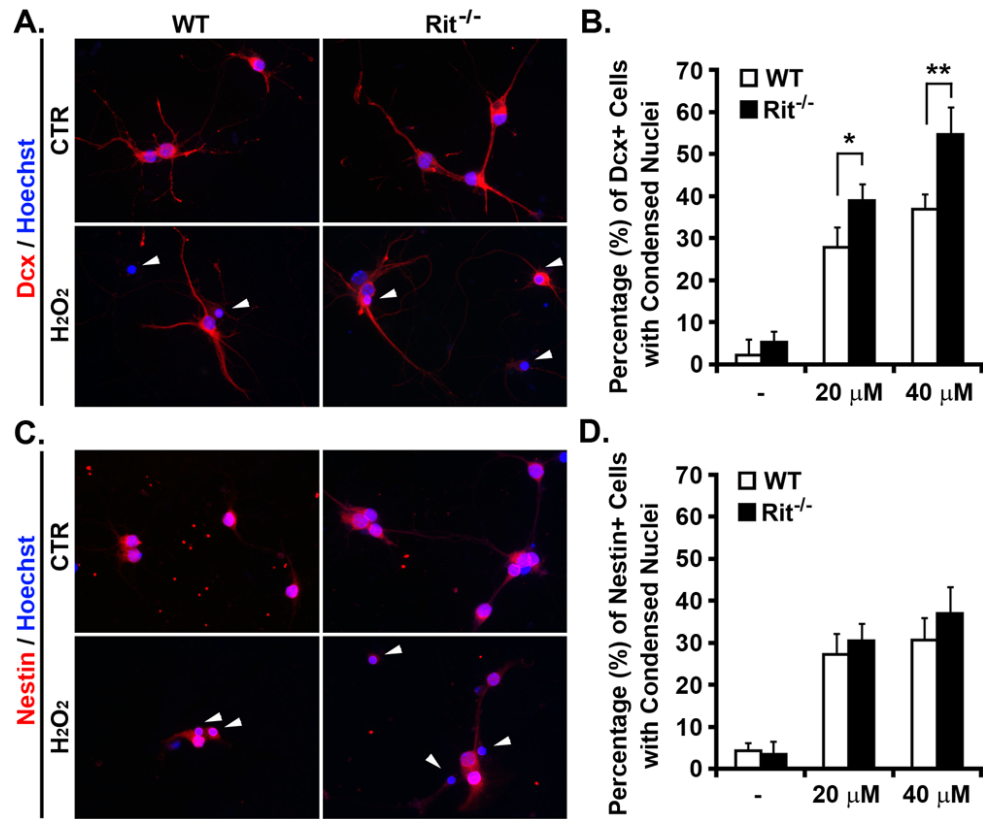
### **Loss of Rit Selectively Sensitizes Doublecortin (Dcx)-Positive Neurons from Hydrogen Peroxide**

A high level of neurogenesis is ongoing within the newborn hippocampus, which inspired us to examine the cellular composition of our *in vitro* cultures. Using biomarkers specific for neurons at different developmental stages, we noted that the vast majority of the cells at DIV8 were identified as either neural stem cells (Nestin<sup>+</sup>) or immature neurons (Dcx<sup>+</sup>), with almost no cells identified in staining for mature neurons (NeuN<sup>+</sup>) (Fig. 5.4A, B). The composition of these cultures were not affected by Rit loss, equivalent percentages of Nestin<sup>+</sup> (WT: 52.7% ± 3.2%, Rit<sup>-/-</sup>: 61.8% ± 5.9%, n = 4, p > 0.1) and Dcx<sup>+</sup> (WT: 57.1% ± 11.0%, Rit<sup>-/-</sup>: 61.32% ± 3.81%, n = 4, p > 0.1) neurons were seen wild-type and Rit<sup>-/-</sup> mice (Fig. 5.4C).

Since Rit loss sensitizes only a fraction of the cultured hippocampal neurons to H<sub>2</sub>O<sub>2</sub> exposure (Fig. 5.2), we next asked whether this might arise in part because Rit signaling contributes to the survival of only a subset of cultured neurons. To explore this possibility, wild-type and Rit<sup>-/-</sup> hippocampal neurons (DIV8) were left untreated or subjected to H<sub>2</sub>O<sub>2</sub> (20 μM and 40 μM for 2 h), neurons fixed, subjected to anti-Nestin (neural stem cell) or anti-Dcx (immature neuron) immunostaining, and apoptotic neurons identified by Hoechst staining (Fig. 5.5A, C). As expected, following H<sub>2</sub>O<sub>2</sub> exposure both Nestin<sup>+</sup> and Dcx<sup>+</sup> neurons undergo apoptosis in a dose-dependent manner (Fig. 5.5B, D). However, while Rit loss dramatically sensitizes Dcx<sup>+</sup> immature neuron to oxidative stress (40 μM H<sub>2</sub>O<sub>2</sub>, WT: 36.8% ± 3.4%, Rit<sup>-/-</sup>: 54.6% ± 6.6%, n = 5, p < 0.01), the survival of Nestin<sup>+</sup> neural stem cells was not affected (40 μM H<sub>2</sub>O<sub>2</sub>, WT: 30.6% ± 5.3%, Rit<sup>-/-</sup>: 36.9% ± 6.4%, n = 4, p > 0.1) (Fig. 5.5B, D). Taken together, these data suggest that Rit signaling selectively promotes Dcx<sup>+</sup> immature neuron survival following oxidative stress.



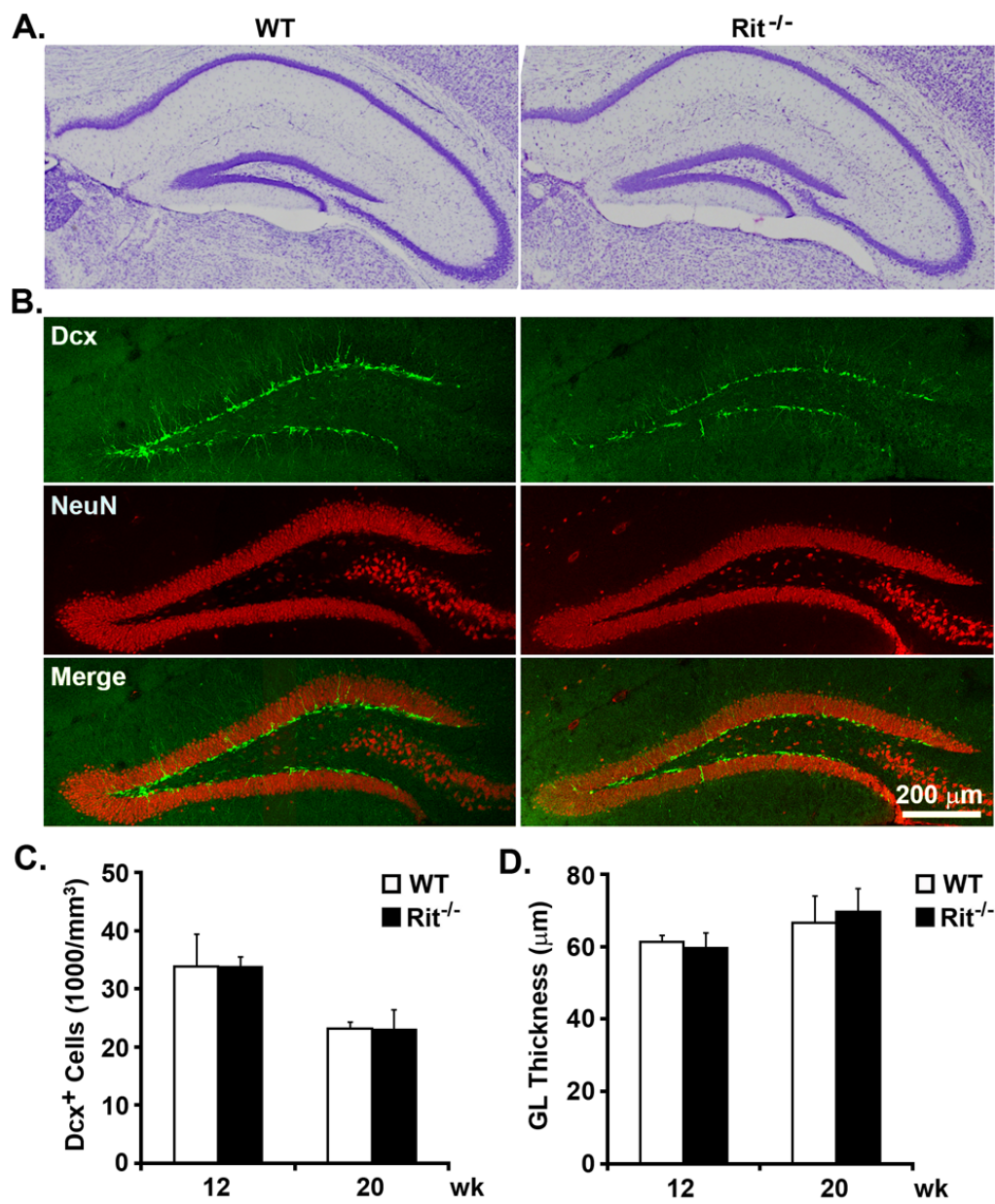
**Figure 5.4 Cellular Composition of *in vitro* Hippocampal Neuron Cultures.** (A, B) *Rit*<sup>-/-</sup> and WT hippocampal neurons were cultured *in vitro* for 8 days (DIV8). Cells were then fixed and immunostained with the indicated antibodies. Anti-Dcx: immature neurons, anti-Nestin: neural stem cells, anti-NeuN: mature neurons. Nuclei were visualized by Hoechst stain. *Panel A and B* show representative images from *Rit*<sup>-/-</sup> neurons. Arrowheads highlight Dcx<sup>+</sup>/Hoechst immature neurons and Nestin<sup>+</sup>/Hoechst neural stem cells. (C) Percentage of Nestin positive and Dcx positive neurons from DIV8 *in vitro* cultured WT and *Rit*<sup>-/-</sup> hippocampal neurons. The data is presented as mean  $\pm$  S.D. (*t*-test, *n* = 4)



**Figure 5.5 Loss of Rit Sensitizes Dcx<sup>+</sup>, but not Nestin<sup>+</sup> Neurons from Oxidative Stress.** (A, C) Rit<sup>-/-</sup> and WT hippocampal neurons were cultured *in vitro* for 8 days (DIV8). On the day of experiment, neurons were left untreated or treated with H<sub>2</sub>O<sub>2</sub> (20 μM and 40 μM H<sub>2</sub>O<sub>2</sub>, 2 h) and immunostained with the indicated antibodies (anti-Dcx or anti-Nestin). Nuclei were visualized with Hoechst stain. *Panel A and C* show representative images of neurons with or without H<sub>2</sub>O<sub>2</sub> treatment. Apoptotic cells are indicated with an arrowhead. (B, D) Percentage of Dcx<sup>+</sup> or Nestin<sup>+</sup> cells with condensed nuclei following oxidative stress. 10-15 fields were randomly selected for each treatment and scored for condensed nuclei. Note that the loss of Rit only increases the apoptosis in Dcx<sup>+</sup> immature neurons. The results are presented as mean ± S.D. (*t*-test: \* *p* < 0.05, \*\* *p* < 0.01, *n* ≥ 3)

### **Loss of Rit Does Not Impair Dentate Gyrus Morphogenesis**

In the adult brain, new neurons are constantly born in the SGZ of the hippocampus. During the maturation process, only the newborn immature neurons that functionally integrate into the existing hippocampal neural circuitry survive, while the majority of the immature neurons undergo apoptosis. As a result, the rate of adult neurogenesis in the hippocampus is under tight control. Since Rit promotes survival signaling in newborn immature neurons, we next examined the effect of Rit loss on dentate gyrus granular layer. Rit loss did not result in any gross morphological changes in hippocampus (Fig. 5.6A), or any obvious broader developmental defects, because  $Rit^{-/-}$  mice had normal body and brain size at all ages examined. The density and size of  $Dcx^{+}$  immature neurons in the DG are similar between  $Rit^{-/-}$  mice and wild-type littermates at two different ages (12 and 20 weeks) (Fig. 5.6B, C). The average thickness of granular layer of the DG was also unchanged, in keeping with a consistent dentate gyrus granular layer volume and similar granular neuron size or density between the wild-type and  $Rit^{-/-}$  animals (Fig. 5.6B, D). Taken together, these data indicate that Rit expression is not required for normal structural development of the dentate gyrus.

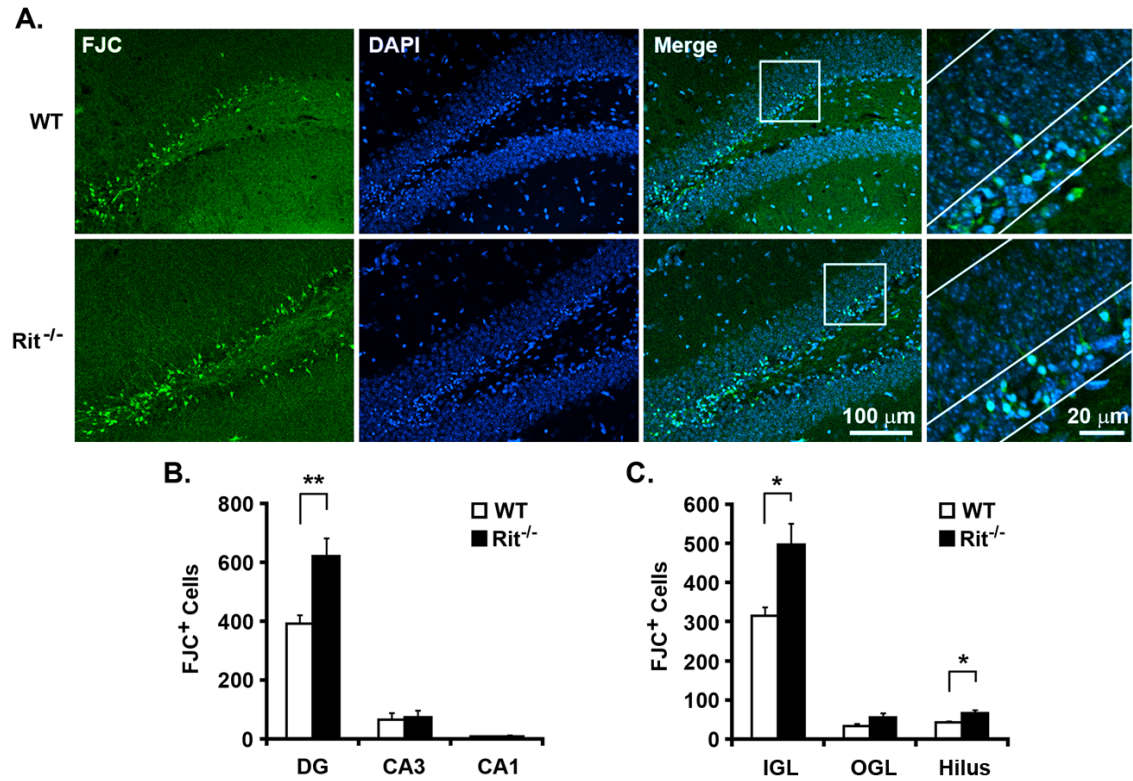




**Figure 5.6 Loss of Rit Does Not Impact Global Brain Morphology or Dentate Gyrus Morphogenesis.** (A) Representative images of Nissl-stained DG sections from 12-week-old and 20-week-old  $Rit^{-/-}$  mice and wild-type mice. (B) Representative images of the dentate gyrus from  $Rit^{-/-}$  mice and wild-type (12 weeks old) immunostained for Dcx (green) and NeuN (red). (C) Quantitative analysis revealed that the number of  $Dcx^{+}$  immature neurons (12 and 20 weeks old) has not altered in the absence of Rit expression. The results are presented as mean  $\pm$  S.D. ( $t$ -test,  $n = 4$ ). (D) Quantitative analysis revealed that the average thickness of granular layer (12 and 20 weeks old) has not changed in the absence of endogenous Rit. The results are presented as mean  $\pm$  S.D. ( $t$ -test,  $n = 4$ ).

### **Rit<sup>-/-</sup> Mice Have Significantly More Degenerated Neurons in the Inner Granular Layer of the Dentate Gyrus following CCI**

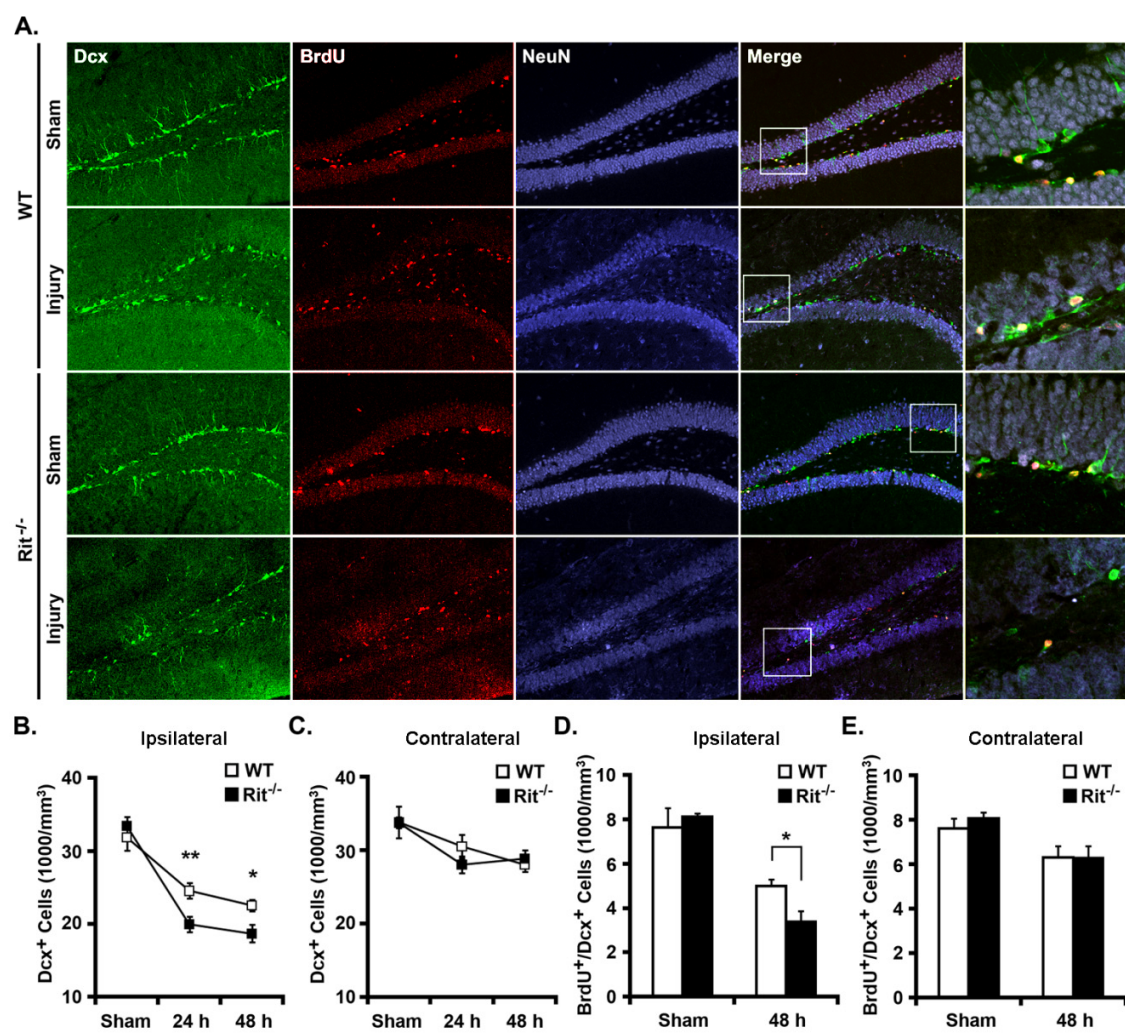
Despite the absence of any defect in basal hippocampal neurogenesis of the SGZ in Rit<sup>-/-</sup> mice, we next examined whether lack of Rit expression affected the survival of immature neurons following controlled cortical impact (CCI) injury, an experimental model of traumatic brain injury (TBI). Contusive TBI is characterized by an initial mechanical disturbance and the activation of subsequent secondary injury cascades, including excitotoxicity and oxidative stress, leading to the selective and acute death of immature neurons in the hippocampal dentate gyrus. Fluorojade-C (FJC) staining was used to examine neuron degeneration 24 h after CCI. Consistent with the previous reports, CCI caused massive neuron degeneration in DG rather than in CA1 and CA3 regions of the hippocampus, as reflected by the increase in FJC-positive neurons (Fig. 5.7A, B). Quantification of FJC-positive neurons within each subregion of the hippocampus revealed that the majority (~80%) of degenerating neurons were located within the inner granular layer (including SGZ and inner one-third of the granular layer) of the dentate gyrus, where immature neurons and neural stem cells reside, with many fewer FJC-positive neurons (~10%) observed in the outer granular layer or hilus each (Fig. 5.7A, *right two columns*, and C). More importantly, Rit<sup>-/-</sup> mice displayed almost twice the number of FJC-positive cells in the DG (WT, n = 9: 391.2 ± 30.2, Rit<sup>-/-</sup>, n = 7: 620.4 ± 62.2, p < 0.01) (Fig. 5.7B), with the majority located within the SGZ (WT, n = 9: 314.7 ± 23.1, Rit<sup>-/-</sup>, n = 7: 497.6 ± 52.6, p < 0.05) (Fig. 5.7C). We also noted in DAPI co-stained sections a concentration of cells with condensed nuclei within the SGZ of DG following CCI (Fig. 5.7A, *column 2 and 4*). These data agree with earlier studies indicating that moderate CCI injury results in selective secondary neuronal death in the hippocampal dentate gyrus, and suggests that Rit signaling plays a neuroprotective role, promoting the selective survival within the dentate gyrus SGZ.



**Figure 5.7 *Rit*<sup>-/-</sup> Mice Have Significantly More Fluorojade-C Positive Neurons in the Dentate Gyrus following Controlled Cortical Impact (CCI) Injury.** (A) Fluorojade-C (FJC) staining was used to identify degenerating neurons in 12-week-old *Rit*<sup>-/-</sup> mice and WT littermates 24 h after being subjected to CCI injury. Nuclei were visualized with DAPI stain. Representative images of dentate gyrus are presented. Note that the majority of FJC<sup>+</sup> cells, and cells with condensed nuclei, are located in the inner granular layer of the DG. (B) Quantification of FJC positive cells the hippocampus. Note that *Rit*<sup>-/-</sup> mice contain significantly more degenerating neurons within the DG. The results are presented as mean  $\pm$  SEM (WT, n = 9; *Rit*<sup>-/-</sup>, n = 7, *t*-test: \*  $p < 0.05$ , \*\*  $p < 0.01$ ) (C) Quantification of FJC positive cells in the subregions of the hippocampal dentate gyrus, which include inner granular layer, outer granular layer and hilus. Note that most FJC<sup>+</sup> cells are located in the inner granular layer of DG and that *Rit*<sup>-/-</sup> mice have significantly more degenerating neurons in the inner granular layer. The results are presented as mean  $\pm$  SEM (WT, n = 9; *Rit*<sup>-/-</sup>, n = 7, *t*-test: \*  $p < 0.05$ , \*\*  $p < 0.01$ )

### **Rit Promotes Newborn Immature Neuron Survival following CCI**

There are a collection of cell types within the hippocampal dentate gyrus, including neural stem cells, newborn immature granular neurons, and mature neurons [148]. These neurons display distinct characteristics, including morphology, expression of cell type-specific proteins, electrophysiological properties, and neural functions. To determine whether Rit promotes neural-subtype selective *in vivo* survival within the dentate gyrus following CCI, 11-week-old *Rit*<sup>-/-</sup> mice and wild-type littermates were subjected to daily intraperitoneal (i.p.) injections with 5-bromodeoxyuridine (BrdU, 50 mg/kg) to label proliferating neuronal stem/progenitor cells (NPCs) for one week prior to CCI. The survival of total immature neurons and newborn BrdU-labeled neurons was estimated by co-staining with the immature neural marker doublecortin (Dcx). In keeping with the lack of obvious alterations in dentate gyrus structure, sham animals showed equivalent numbers of Dcx<sup>+</sup> immature neurons in the DG of *Rit*<sup>-/-</sup> mice and wild-type littermates. CCI injury led to the loss of Dcx<sup>+</sup> neurons in the DG of the ipsilateral hemisphere 24 h and 48 h post-injury in wild-type mice (~30% cell loss at 48 h), with only a small decrease in the contralateral dentate gyrus. However, *Rit*<sup>-/-</sup> mice display a dramatically more severe loss in Dcx<sup>+</sup> neurons post injury (24 h, WT, n = 9:  $24.5 \pm 1.1 \times 10^3 / \text{mm}^3$ ; *Rit*<sup>-/-</sup>, n = 7:  $19.9 \pm 1.1 \times 10^3 / \text{mm}^3$ ,  $p < 0.01$ ; 48 h, WT, n = 10:  $22.5 \pm 0.8 \times 10^3 / \text{mm}^3$ ; *Rit*<sup>-/-</sup>, n = 9:  $18.7 \pm 1.2 \times 10^3 / \text{mm}^3$ ,  $p < 0.05$ ) (Fig. 5.8A-C). Similarly, when we assessed BrdU/Dcx double positive cell numbers 48 h post-CCI injury, the density of BrdU<sup>+</sup>/Dcx<sup>+</sup> neurons within the dentate gyrus decreased in wild-type mice, and the loss in BrdU<sup>+</sup>/Dcx<sup>+</sup> neuronal density was significantly greater in *Rit*<sup>-/-</sup> mice (48 h, WT, n = 10:  $5.0 \pm 0.3 \times 10^3 / \text{mm}^3$ ; *Rit*<sup>-/-</sup>, n = 9:  $3.4 \pm 0.5 \times 10^3 / \text{mm}^3$ ,  $p < 0.05$ ) (Fig. 5.8 A, D-E). Taken together, these data indicate that Rit signaling is critical for the protection of newborn immature neurons in DG following CCI.



**Figure 5.8 Loss of Rit Impairs Newborn Immature Neuron Survival following CCI.**

(A) 11-week-old  $Rit^{-/-}$  mice and wild-type littermates were injected (i.p.) with BrdU (50 mg/kg) daily for one week, and mice subjected to CCI 2 days after the last injection. Brain sections from  $Rit^{-/-}$  and wild-type littermates were stained with Dcx and BrdU antibodies. Note that Dcx/BrdU double positive cells (arrowhead) represent newborn immature neurons produced during the 9 days prior to injury. (B, C) Total Dcx positive cells in the DG in both contralateral and ipsilateral hemispheres following CCI were quantified and cell densities calculated. The results are presented as mean  $\pm$  SEM (WT, sham, n = 7; 24 h, n = 9; 48 h, n = 10;  $Rit^{-/-}$ , sham, n = 6; 24 h, n = 7; 48 h, n = 9, *t*-test: \*  $p < 0.05$ , \*\*  $p < 0.01$ ). (D, E) BrdU/Dcx double positive cells within the contralateral and ipsilateral DG were quantified following CCI and cell densities calculated. The results are presented as mean  $\pm$  SEM. (WT, sham, n = 7; 48 h, n = 10;  $Rit^{-/-}$ , sham, n = 6; 48 h, n=9, *t*-test: \*  $p < 0.05$ ).

## DISCUSSION

In this chapter, we examine the physiological role for Rit signaling in the central nervous system and find that Rit loss results in increased apoptosis in cultured primary hippocampal neurons in response to oxidative stress, particularly Dcx<sup>+</sup> immature neurons, supporting a pro-survival role for Rit-dependent signaling in response to oxidative damage. Analysis of these cultures indicates that impaired activation of p38 and Akt signaling in Rit<sup>-/-</sup> neurons following ROS contributes to the increase in cell death, as first seen in primary MEFs (see Chapter Three), supporting a conserved role for the p38-MK2-HSP27-Akt cascade signaling in Rit-mediated survival.

More importantly, in keeping with our findings using *in vitro* cultured neurons, traumatic brain injury leads to increased newborn immature neuron loss in the DG of Rit<sup>-/-</sup> mice when compared to wild-type littermates. These studies extend our understanding of Rit-mediated protection to neurons, and suggest that Rit may play a critical role in the survival of adult newborn hippocampal neurons, and thus may play a general role in adult neurogenesis, and specifically in the cellular repair and regenerative mechanisms that have been implicated in the cellular remodeling that occurs following various types of brain injury. Exposure to reactive oxygen species (ROS) is inevitable in aerobic organisms, as it is produced in mitochondria as a by-product of the electron transport chain. Cells have developed elaborate defense mechanisms, including enzymes such as superoxide dismutases, catalases, and peroxidases, as well as small molecule antioxidants such as glutathione [189]. Physiological levels of ROS are beneficial and have been found to regulate a diverse array of biological activities including cell migration, proliferation and differentiation [193, 212, 214]. However, the accumulation of intracellular ROS beyond the cell's antioxidant capacity is detrimental. These highly reactive small molecules are capable of disrupting the normal function of proteins and lipids by oxidation, resulting in DNA lesions, and eventually resulting in levels of cellular damage sufficient to initiate programmed cell death (apoptosis). This is particularly true in neurons because of their relatively low antioxidant capacity. Indeed, oxidative stress is one of the major causes of neuronal loss following stroke, seizure, brain trauma, and in certain neurodegenerative diseases including Alzheimer's and Parkinson's disease [190].

In this chapter, we find that Rit appears to control a crucial pro-survival pathway to protect newborn immature hippocampal neurons from oxidative stress. These data are, to our knowledge, the first to prove a key role for a small GTPase in the survival of a select subpopulation of developing neurons and also implicate a regulatory function for Rit in adult hippocampal neurogenesis, particularly in the case of immature hippocampal neurons following traumatic brain injury. The detailed molecular mechanism of neural survival, however, remains unclear. Impaired phosphorylation of p38 MAPK and Akt in the absence of Rit following H<sub>2</sub>O<sub>2</sub> exposure, coinciding with increased apoptosis, suggests Rit-mediated pro-survival pathway may involve in the previously characterized Rit-p38-MK2-HSP27-Akt signaling cascade in cell lines and MEFs [112, 183]. However, more experiments are required to confirm a role for the p38-MK2-HSP27-Akt signaling cascade in Rit-mediated pro-survival mechanisms in neurons. Furthermore, we cannot rule out the possibility of the existence of other protective pathways downstream of Rit. As discussed in the previous chapters, p38 MAPK has been identified as upstream regulator of multiple pro-survival pathways, including MSK1/2-CREB, and GSK3 $\beta$ - $\beta$ -catenin signaling [224, 225]. To fully dissect the Rit-mediated pro-survival signaling pathway(s) in neurons, an additional transgenic mouse expressing constitutively active Rit exclusively in neurons is needed. We expect that active Rit expressed neurons, especially in newborn immature neurons, would promote resistance to oxidative stress and traumatic brain injury. More importantly, using specific pharmacological inhibitors, we will be able to critically examine the contribution of putative downstream components of Rit-mediated protective pathways. In addition, it would also be interesting to ask whether Rit signaling is important in other types of neurons in the central nervous systems. The SVZ is another highly active neurogenesis niche in the brain containing a large number of newborn immature neurons [136]. Whether Rit also plays critical roles in immature neuron survival in SVZ following stresses and brain injuries awaits to be addressed.

Traumatic brain injury is known to result in a massive acute loss of neural stem cells and immature neurons in the hippocampal dentate gyrus, the very source of cells needed to supply new hippocampal granule neurons to promote recovery and repair [148, 173, 175]. Compromising the pool of newborn immature neurons exacerbates, in the long



term, the neuron loss and cognitive dysfunction following TBI [181, 182]. Therefore, identifying immature neuron protective mechanisms is of clinical importance in the treatment of traumatized patients. In the present chapter, both the *in vitro* and *in vivo* studies revealed that Rit selectively promotes Dcx<sup>+</sup> newborn immature neuron survival following oxidative stress. It is plausible that selective activation of Rit-mediated pro-survival signaling in a short window after TBI would significantly decrease the loss of Dcx<sup>+</sup> immature neurons, lead to a faster-than-normal recovery, and subsequently, ameliorate the cognitive dysfunction suffered from TBI. Generation of a transgenic mouse allowing selective active Rit expression in immature neurons will allow us to test this notion.

In conclusion, the data presented in this chapter extends our understanding of the physiological role of Rit to the central nervous system. Rit selectively promotes adult born immature neuron survival following oxidative stress and traumatic brain injury. This protective effect likely depends upon p38 MAPK-MK2-HSP27-Akt signaling, and it will be interesting to determine whether the mTORC2 complex is also involved in Akt survival signaling in neurons. Oxidative stress is one of the major mechanisms underlying neuron loss in a collection of brain injuries and neural disorders [190]. In addition to brain trauma, stroke and seizure, two of the most common injuries to the brain, also lead to lethal ROS accumulation. Meanwhile, in neurodegenerative disorders including Alzheimer's disease and Parkinson's disease, toxic levels of ROS have also been seen. A greater understanding of Rit signaling, and the biological control of adult neurogenesis is likely to provide crucial insight into the etiology and potential therapeutic interventions of major brain disorders. It will be interesting in future to determine whether Rit plays a similar pro-survival role in these injury and disease models, and subsequently whether Rit-mediated signaling pathways could be targeted for therapeutic treatment of these neuronal disorders.

## CHAPTER SIX

### Rit Controls IGF-Mediated Signaling

#### INTRODUCTION

Receptor tyrosine kinases (RTKs) are a class of biologically critical receptors involving in mitogen signaling to control a wide spectrum of cellular processes including cell growth, differentiation and survival [249, 250]. Generally, the activation of receptor tyrosine kinases requires the ligand binding on the extracellular domains. Each ligand simultaneously binds to the extracellular domain of two RTKs, which leads to the dimerization of receptors. The dimerization has been proven critical for the activation of the receptor by triggering the trans-autophosphorylation of tyrosine residues located within the intracellular tail domain of the receptors [249]. These critical phosphorylated tyrosine residues serve as the docking sites for a collection of Src homology (SH2) domain-containing adapter proteins, which in turn recruit additional proteins/kinases [251, 252]. The generation of this ligand-mediated dynamic signaling complex allows RTKs to regulate multiple downstream signaling pathways including MAPK cascades and PI3K-Akt pathway [249].

IGF-1 (insulin-like growth factor-1) is a 70 amino acid polypeptide which regulates cellular growth and energy metabolism through its binding and activation of IGF-1 receptor (IGF-1R) [253]. Structurally similar to insulin, IGF-1 has 2 chains connected by disulphide bond. IGF-1 is mainly produced in liver and delivered to the target organs by circulation, although a raising body of evidence also indicates that IGF-1 can be made locally in the target tissues to act in a paracrine fashion to modulate IGF-1 mediated biological functions [254]. IGF-1 signaling plays key roles in multiple biological processes [162, 253-256]. IGF-1 stimulation elicits survival and proliferation signals which are particularly important during development. Loss of functional IGF-1 signaling in both humans and animal models results in a significantly smaller body size [253, 255]. Paradoxically, IGF-1 signaling also potentiates the aging process [256]. Impaired IGF-1 leads to an extended lifespan in *C.elegans*. Similar phenomena are also observed in mammals, including humans, suggesting the evolutionary conserved role of IGF-1 signaling in aging/longevity. One explanation for this contribution to aging is that

impairment of the IGF-1/Akt signaling axis results in activation of the transcription factor FOXO, which controls transcription of a set of longevity genes including antioxidant enzymes [257]. Moreover, IGF-1 signaling can elicit anti-inflammatory pathways via Akt-IKK-NF $\kappa$ B signaling to prevent chronic inflammatory diseases such as atherosclerosis [258, 259].

Recently, IGF-1 has been shown to play important roles in the central nervous system [167]. IGF-1 can be produced locally in the brain predominantly by microglia or from the circulation by crossing the blood-brain barrier [161, 165, 167]. Besides its protective effects on neurons, IGF-1 has been shown to induce proliferation and differentiation of neural stem cells/neural progenitor cells *in vitro* and *in vivo* [167]. Interestingly, IGF-1 appears to selectively induce the proliferation of neural progenitor cells in the hippocampal dentate gyrus and their subsequent differentiation into neurons rather than glial cells [161, 167]. During this process, ERK signaling appears particularly important, as pharmacological inhibition of MEK/ERK pathway has been shown to abolish IGF-1-induced neural stem cell proliferation [165-167].

Ras GTPases are critical downstream targets of RTKs including IGF-1 receptor. Upon activation, RTKs are capable of recruiting guanine nucleotide exchange factors via adapter proteins and promote the activation of Ras family GTPases, which then serve to couple RTK signaling to PI3K-Akt and MAPK cascades among others [254, 260]. The functional significance of the “orphan” GTPase Rit on RTK signaling is only partially characterized. Our previous studies have shown that Rit plays roles in coupling NGF-TrkA signaling to ERK and p38 signaling to control neurite outgrowth in PC6 cells [95]. This role of Rit is extended to EGF/EGF receptor-mediated signaling by the studies transiently knocking-down Rit in PC6 and HeLa cell [112]. However, whether Rit plays a similar role for other RGK signaling in other cell types, particularly primary cells, remains to be determined.

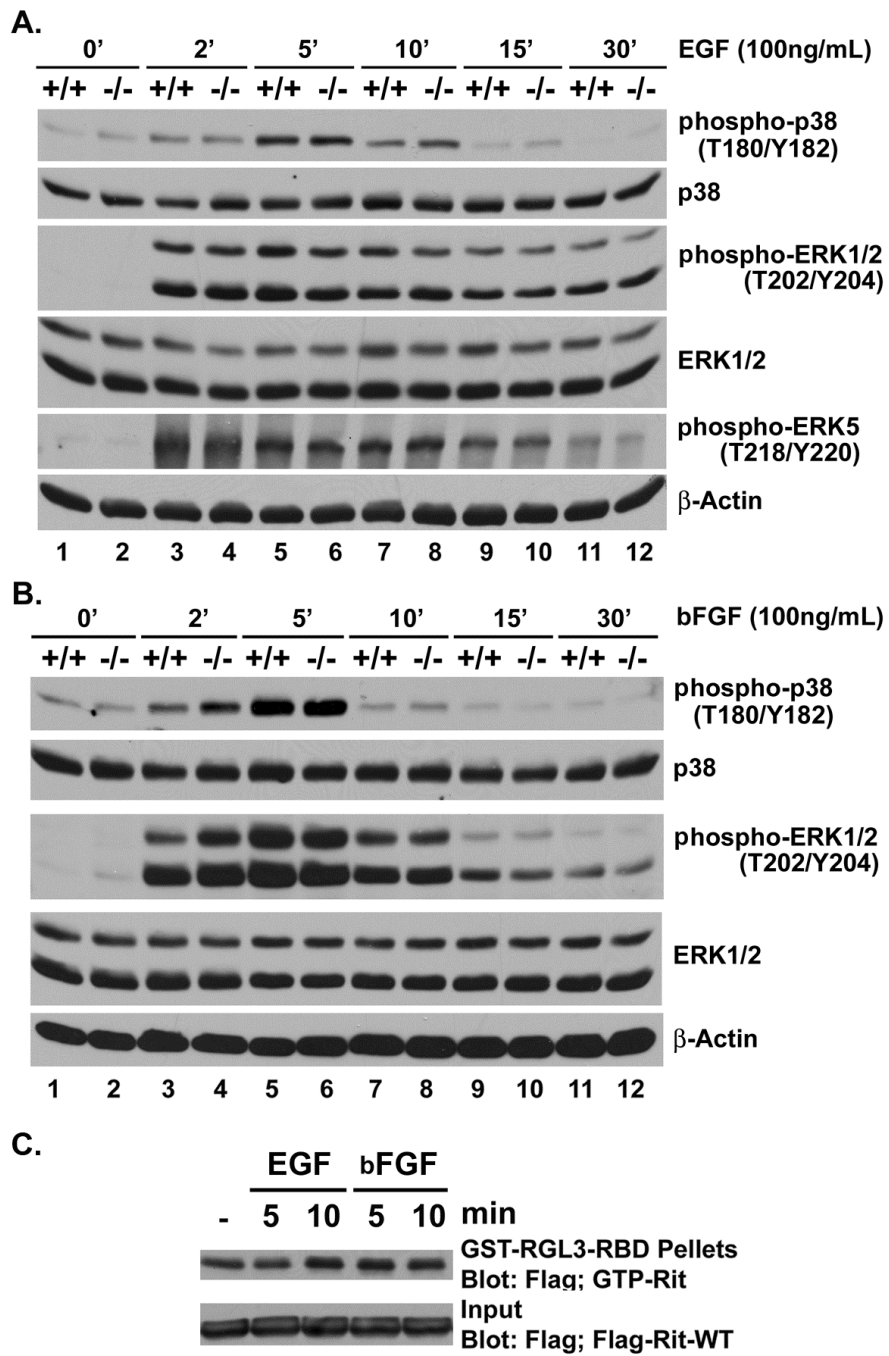
Here, in this chapter, we examined the role of Rit small GTPase in distinct RTK signaling in both primary MEFs and primary hippocampal neurons using our genetically engineered Rit-null mice. We find that Rit loss results in severe disruption of ERK signaling in response to IGF-1 but surprisingly not to EGF, bFGF or BDNF stimulation. These studies indicate that Rit is critical in linking IGF-1R activation to ERK signaling in

both primary MEFs and primary hippocampal neurons, and suggest that Rit plays a previously unsuspected role in IGF-1 mediated biological activities, including regulation of adult neurogenesis.

## **RESULTS**

### **Rit Loss Has No Effect on EGF- and bFGF-Mediated Signaling**

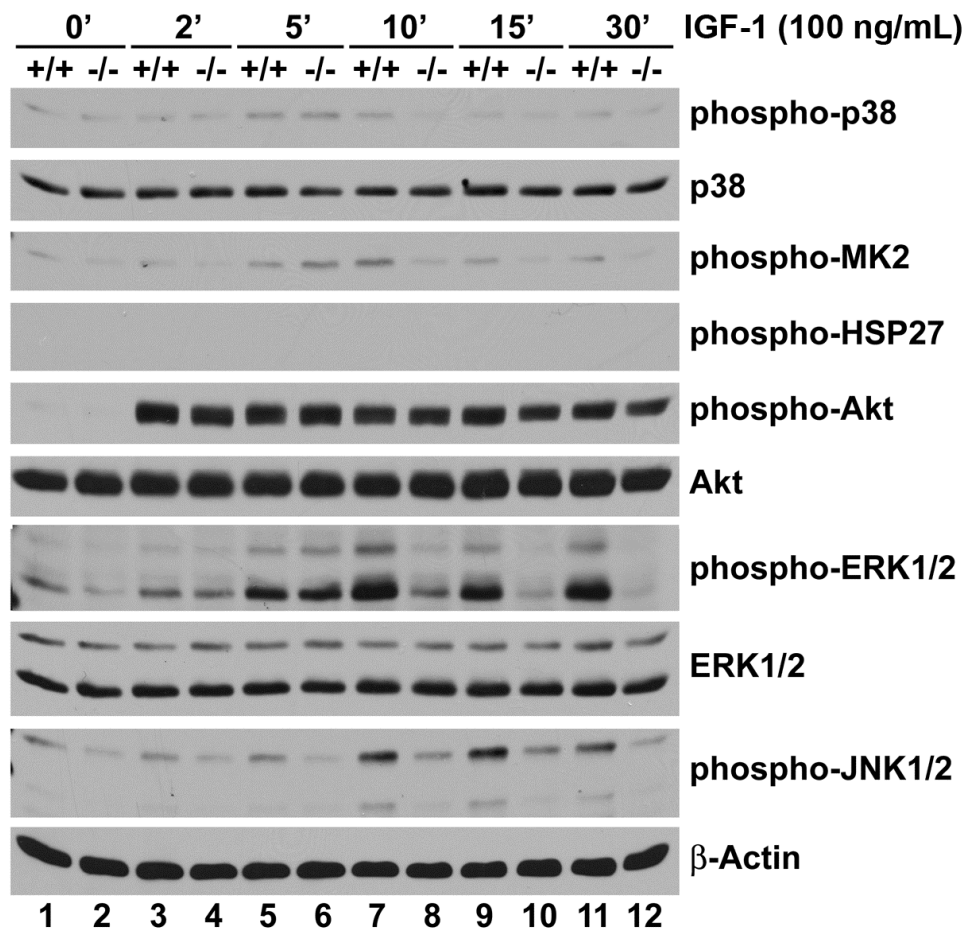
We have previously shown using shRNAi silencing methods that Rit signaling plays a critical role in coupling both NGF (via the receptor tyrosine kinase TrkA) and PACAP38 (via GPCR signaling) to a variety of MAP kinase cascades in pheochromocytoma cells [95, 108]. To assess the contribution of Rit to mitogen-mediated intracellular signaling, we made use of primary mouse embryonic fibroblasts (MEFs) isolated from Rit null mice as our model system. Primary MEFs derived from Rit<sup>-/-</sup> mice and wild-type littermates were serum starved for 3 h followed by the stimulation of 100 ng/mL EGF or bFGF for indicated time duration (Fig. 6.1A,B). Stimulation with these growth factors led to potent activation of MAPK cascades including ERK1/2/5 and p38 MAPK. Surprisingly, Rit does not appear critical in EGF and bFGF-mediated MAPK signaling, since Rit deficiency has no obvious effects on the phosphorylation of MAPKs (Fig. 6.1A,B). We next asked whether Rit is activated by EGF and bFGF stimulation using Rit-GTP pull-down assay in MEFs transiently transfected with 3×Flag-Rit-WT (Fig. 6.1C). Stimulation of EGF and bFGF led to the elevation of GTP-bound Rit levels. Taken together, despite the finding that growth factors such as EGF and bFGF are capable of stimulating Rit GTP loading, Rit is dispensable in EGF and bFGF signaling in primary MEFs.



**Figure 6.1 Rit Is Not Essential for EGF and bFGF-mediated signaling.** (A-B) Lysates from wild-type and Rit<sup>-/-</sup> MEFs were prepared following EGF and bFGF exposure (100 ng/ml) for the indicated times and analyzed by immunoblotting with the indicated antibodies. (C) Immortalized wild-type MEFs transfected with Flag-Rit-WT were serum starved for 3 h, prior to stimulation with EGF (100 ng/ml) and bFGF (100 ng/ml) for the indicated times, and GTP-bound Rit recovered by GST-RGL3-RBD pull down. Precipitated GTP-Rit was detected by anti-Flag immunoblotting.

### **Loss of Rit Impairs ERK and JNK Signaling in Response to IGF-1 in Primary MEFs**

We next assessed the contribution of Rit to IGF-1/IGF1R signaling, another well-defined RTK signaling (Fig. 6.2). As expected, IGF-1 stimulation on serum-starved primary MEFs triggered robust phosphorylation of Akt within 2 min after initial exposure. Accompanied with the rapid Akt activation, IGF-1 stimulates decent activation of ERK1/2, however, with a much delayed kinetics. Moreover JNK1/2 was also activated modestly upon IGF-1 stimulation. In contrast, IGF-1 failed to activate p38 MAPK, which was further confirmed by the phospho-blotting of MK2 and HSP27, two of the well-known p38 targets. More importantly, Rit appears critical for the activation of the selective downstream signaling molecules tested here. Rit loss leads to severely impaired ERK1/2 and JNK1/2 activation following IGF-1, suggesting the critical role of Rit in IGF-1-ERK/JNK signaling. Akt phosphorylation, however, was not significantly altered in the absence of Rit, despite the modest decrease at later time points (15 min and 30 min), indicating that Rit may play some minor roles in the duration of IGF-1 mediated Akt signaling. Collectively, different Rit dependency in EGF, bFGF and IGF-1 signaling indicates that distinct receptor tyrosine kinases may preferentially utilize different small GTPases to control MAPK signaling. Rit may, therefore, selectively contribute to IGF-1-mediated, ERK-dependent biological activities including survival and proliferation.

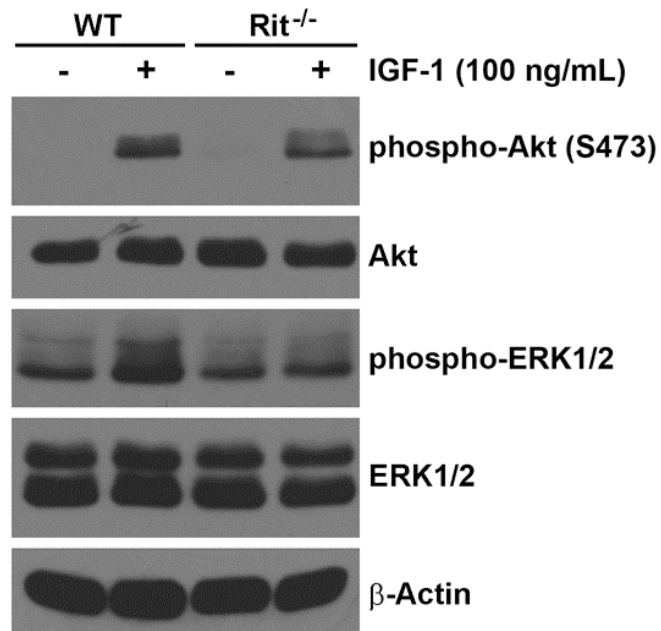


**Figure 6.2 Rit Loss Selectively Impairs ERK and JNK Signaling following IGF-1 Stimulation.** Primary MEFs (P4) derived from Rit<sup>-/-</sup> mice and wild-type littermates were serum starved for 3 h prior to 100 ng/mL IGF-1 treatment for indicated times. Total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies.



### **Rit Deficiency Severely Attenuates IGF-1 Mediated Signaling in Hippocampal Neurons**

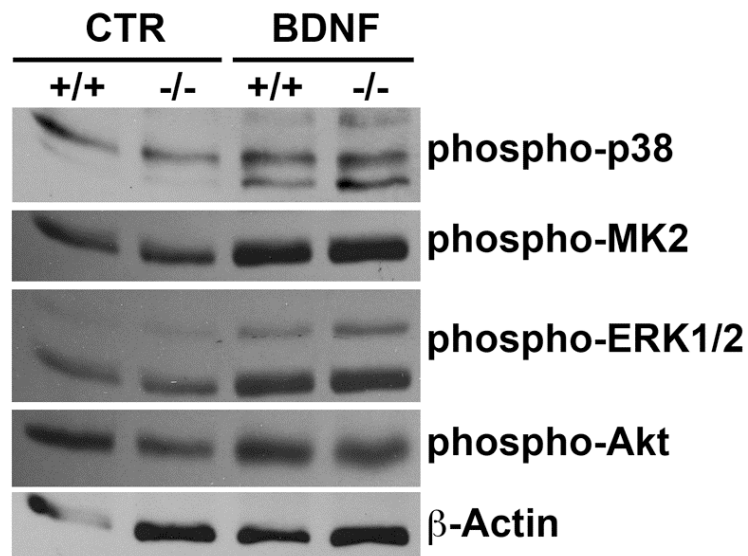
IGF-1 stimulation promotes neural stem cell proliferation and differentiation into granule neurons within the dentate gyrus of the hippocampus, suggesting that IGF-1 might play a critical role in the regulation of adult neurogenesis [161, 162, 167]. Indeed, ERK1/2 signaling appears critical to IGF-1 mediated adult neurogenesis in both *in vitro* and *in vivo* models [165, 166]. To examine the contribution of Rit to IGF-1 signaling in neurons, primary hippocampal neurons (DIV8) isolated from Rit<sup>-/-</sup> mice and wild-type littermates were starved in serum-free DMEM for 40 min prior to IGF-1 stimulation for 15 min (Fig. 6.3). As expected, IGF-1 treatment promoted both Akt and ERK1/2 activation, as monitored by anti-phospho-antibody immunoblotting. More importantly, loss of Rit attenuates Akt and, particularly, ERK1/2 MAPK phosphorylation following IGF-1 treatment.



**Figure 6.3 Rit Deficiency Attenuates IGF-1 Mediated Signaling in Hippocampal Neurons.** Rit<sup>-/-</sup> and wild-type hippocampal neurons were cultured for 8 days (DIV8). On the day of experiment, the neurons were starved in serum-free DMEM for 40 min prior to 15 min of IGF-1 (100 ng/mL) exposure. The total cell lysates were prepared and analyzed by immunoblotting with indicated antibodies.

### **Rit Loss Has No Effect on BDNF Signaling in Hippocampal Neurons**

Brain-derived neurotrophic factor (BDNF) receptor, TrkB, is highly expressed in hippocampus, and BDNF-TrkB signaling has also been implicated in the regulation of many critical brain functions including neurogenesis [157, 250]. To examine whether Rit also contributes to BDNF signal transduction, Rit<sup>-/-</sup> and wild-type hippocampal neurons were starved with serum-free DMEM for 40 min followed by the treatment of BDNF for 15 min (Fig. 6.4). Surprisingly, Rit loss has no significant impact on BDNF-mediated ERK1/2, p38 MAPK or Akt phosphorylation. These data suggested that either TrkB does not serve to stimulate Rit activation in hippocampal neurons or that Rit-mediated signaling does not play an essential role in coupling BDNF to ERK, p38, MK2, or Akt signaling.



**Figure 6.4 Rit Loss Does Not Affect BDNF-Mediated Signaling in Hippocampal Neurons.** *Rit*<sup>-/-</sup> and wild-type hippocampal neurons were in vitro cultured for 8 days (DIV8). On the day of experiment, the neurons were starved in serum-free DMEM for 40 min prior to BDNF (50 ng/mL, 15 min) exposure. Total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies.

## DISCUSSION

IGF-1 acts primarily through its receptor, IGF-1R, at physiological concentrations, although IGF-1 is also able to bind to the insulin receptor with a significant low affinity [253]. Stimulation of the IGF-1R results in the activation of multiple signaling cascades, including Ras family small GTPases, which in turn serve to couple IGF1/IGF-1R signaling to the activation of PI3K-Akt and a variety of MAPK cascades including ERK [254, 260]. In the present chapter, we show that Rit is a key player in IGF-1 signaling. Rit loss was found to attenuate both Akt and ERK activation following IGF-1 stimulation in MEFs and the importance of Rit to IGF-1 signaling was even more pronounced in hippocampal neurons. Surprisingly, Rit loss does not affect BDNF signaling in neurons as well as bFGF and EGF signaling in MEFs. Collectively, our data suggest that Rit may function as a central regulator of ERK and Akt activity specifically downstream of IGF-1/IGF-1R, which cannot be compensated by other Ras family proteins. This exciting finding may suggest that IGF-1R selectively utilize Rit GTPase to control ERK and Akt signaling pathways and subsequently IGF-1 mediated physiological activities.

IGF-1 is a potent anabolic and mitogenic factor and is expressed at a particularly high level in embryos and young adults supporting its key roles in development. Deletion of IGF-1 leads to newborn mice that are 30% smaller than their wild-type littermates [253]. In humans impaired IGF-1 signaling leads to Laron syndrome, in which patients suffer severe growth retardation starting in infancy [253]. In adults, IGF-1 signaling has also been proven critical for tissue homeostasis especially in tissues which undergo constant cell renewal. For instance, IGF-1 promotes the proliferation of osteoblasts [261]. Impaired IGF-1 signaling results in unbalanced bone acquisition and maintenance, leading to a greatly increased risk for developing osteoporosis. IGF-1 also enhances fibroblast proliferation to facilitate the process of wound healing [262]. Moreover, IGF-1 has been shown to induce the proliferation of brown adipocytes [263]. IGF-1-induced cell proliferation appears to rely upon the ERK1/2 MAP kinase signaling cascade since blockade of MEK/ERK signaling abrogates the mitogenic effect of IGF-1 in different experiment models including many of those discussed above [261, 263, 264]. The discovery of IGF-1 receptor (IGFR) in neoplastic tissue and cancer leads to the

speculation that IGF-1 signaling may also facilitate and required for neoplasia and cancer development [254]. Indeed, several studies have been reported that elevated IGF-1 levels results in an increased risk of developing cancer [254]. Therefore, a great deal of effort has targeted IGF-1 signaling for cancer prevention and treatment. Our data demonstrating that Rit contributes to IGF-1-mediated ERK activation in different primary cell types suggests that Rit could be involved in the regulation of primary cell proliferation, particularly those cells most sensitive to IGF-1. However, this is not a global defect, since loss of Rit fails to cause any obvious growth retardation during the development [183]. The normal sized Rit<sup>-/-</sup> animals suggest that the reduced activation of ERK signaling in response to IGF-1 may not be sufficient to cause gross developmental defects and can be possibly compensated by other mitogen or growth factor signaling pathways including EGF and bFGF, in which Rit may play a minor role as shown in primary MEFs (Fig. 6.1).

One of the intriguing findings of this chapter is Rit appears to selectively control IGF-1R signaling rather than other receptor tyrosine kinase signaling including EGF receptor, FGF receptor as well as BDNF receptor TrkB. One simple explanation of normal BDNF signaling in the absence of Rit would be BDNF fails to activate Rit in primary hippocampus neurons. However, this is not likely since our data showed that Rit can be activated by a wide collection of extracellular stimuli from mitogens, growth factors to neurotrophins in multiple cell models [95, 108, 109, 111]. The select dependency of Rit in distinct receptor tyrosine kinase signaling may suggest the ability of each type of RTKs to assemble its unique intracellular signaling complex upon stimulation. RTKs activate small GTPases by directly or indirectly recruiting GEFs onto the C-terminal domain of the receptor [254]. It is possible that IGF-1R is capable of more efficiently recruiting Rit-specific GEFs over the ones for other small GTPases. Therefore, the IGF-1-mediated intracellular signaling cascades controlled by small GTPases particularly MAPK cascades may be largely dependent upon Rit but not other small G-proteins. One unique feature of IGF-1R signaling that distinguishes itself from other RTK signaling is the activated receptor recruits and phosphorylates insulin receptor substrate-1 (IRS-1) [167]. As a result, phosphorylated IRS-1 provides an additional series of phospho-tyrosine residues and presents a whole different platform for the IGF-1R

specific protein recruitment, which may be responsible for the key role of Rit in IGF-1 signaling.

In summary, we identified Rit as a critical regulator of IGF-1 signaling particularly in hippocampal neurons. Loss of Rit impairs both Akt and ERK activation in response to IGF-1, indicating the pivotal role of Rit in IGF-1-dependent biological functions including cell survival and proliferation. In addition, these studies promote a provocative hypothesis that Rit-dependent effects on adult hippocampal neurogenesis may involve defects in IGF-1 signaling. However, more studies are warranted to confirm this hypothesis that Rit is a critical regulator of IGF-1 mediated neuron survival and neurogenesis, including a potential role for Rit in modulating recovery from brain injury and in age-related neurodegenerative diseases including Alzheimer's disease.

## CHAPTER SEVEN

### Conclusions and Future Directions

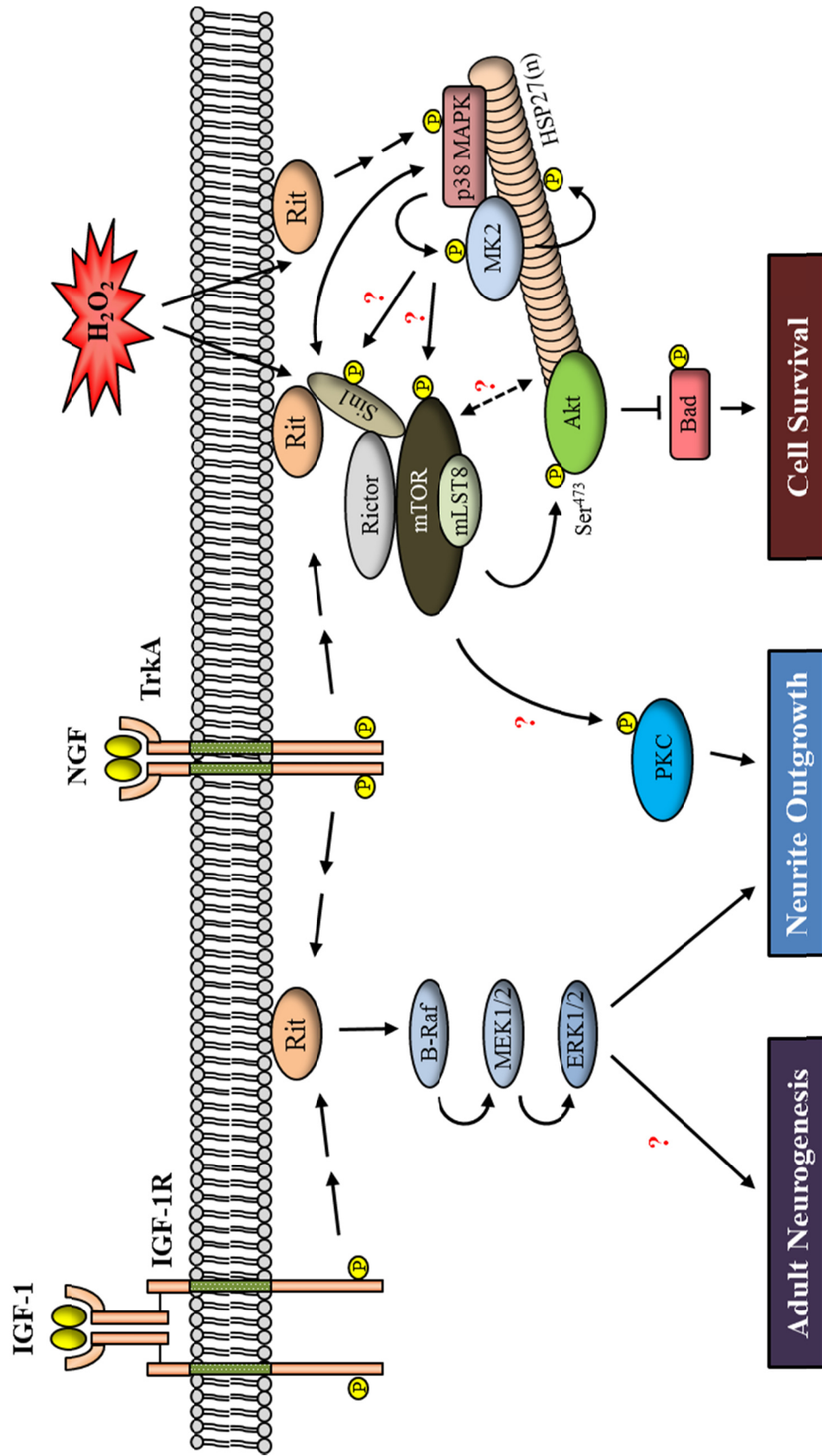
The work presented in this dissertation was performed with the goal of determining the central physiological function of the Rit GTPase and using this molecular insight to explore its cellular roles. In order to address this question, we engineered a mouse line lacking endogenous Rit expression. Using this mouse model we have identified a fundamental role of the Rit GTPase in stress-activated MAPK regulation and pro-survival signaling. Rit deficiency renders both primary fibroblasts and neurons susceptible to apoptosis and leads to a selective disruption of MAPKs and Akt signaling cascades following oxidative stress exposure. The pro-survival effect of Rit is likely due in part to its ability to activate a p38-dependent MK2-HSP27-Akt signaling cascade. In studies that are not included in these thesis (a collaboration with Dr. Jennifer Rudolph, a former Ph.D. student in the Andres laboratory), we have found that Rit signaling is evolutionarily conserved, contributing to stress response signaling in *Drosophila*. Flies lacking the RIC GTPase, a Rit ortholog, are sensitive to a variety of environmental stresses, a phenotype which overlaps those caused by deletion of *D-p38* [183]. Taken together, our studies define a fundamental and evolutionarily conserved signaling link between the Rit GTPase and p38-Akt kinase cascade, revealing a critical pro-survival role for this cascade in cells adapting to oxidative stress.

Cells mobilize diverse signaling cascades to protect against stress-mediated injury. p38 mitogen-activated protein kinase (MAPK) signaling serves as a critical fulcrum in this process, regulating networks that stimulate cellular apoptosis but also have the capacity to promote cell survival. However, relatively little is known concerning this functional dichotomy, particularly the regulation of p38-dependent survival pathways. Our data support a mechanism in which Rit promotes cell survival by directing an unexpected p38 MAPK-dependent Akt survival pathway. Following stress exposure, Rit<sup>-/-</sup> MEFs and hippocampal neurons display increased apoptosis and selective disruption of p38 MAPK signaling, while expression of constitutively activated Rit promotes p38-Akt-dependent cell survival. Our data suggest that Rit is a central regulator of stress-mediated activation of the scaffolded p38-MK2-HSP27-Akt pro-



survival signaling complex, and that survival in part results from activation of this novel cascade. However, a deeper understanding of the control of this pathway, particularly how p38/MK2 promote the activation of Akt remain poorly characterized. Our preliminary studies indicate that the mTORC2 complex is required for p38-Akt directed survival signaling, with Rit-p38 signaling leading to the increased mTORC2 activity, and mTORC2 being essential for Akt stimulation. These exciting data support a critical role for mTORC2 in the cellular oxidative stress response, and implicate the HSP27 regulatory complex in the regulation of mTORC2 signaling.

We have extended our analysis to examine the role of Rit signaling to the central nervous system, and have found that Rit plays a biologically pro-survival effect in a select population of hippocampal neurons. Rit null neurons, particularly *Dcx*<sup>+</sup> newborn immature neurons, are significantly more vulnerable to oxidative stress and traumatic brain injury, indicating that Rit likely plays a crucial in controlling hippocampal neurogenesis, helping to regulate newborn neural survival in response to environmental stress and injury. Since the continuous generation and integration of hippocampal neurons appears critical for the maintenance of learning and memory, and a majority of newborn neurons undergo apoptosis at early stages of development in response to a poorly understood network of both endogenous and exogenous factors [151], these studies might simply started to identify the functional role for Rit in neurogenesis. Moreover, our results have also shown that Rit plays a selective role in IGF-1-mediated signaling, particularly ERK activation. We find that IGF-1-mediated downstream signaling is severely impaired in *Rit*<sup>-/-</sup> neurons and MEFs, but that other RTK signaling, including EGF, FGF, and BDNF do not appear to be altered. These data support an important role of Rit in IGF-1-mediated cellular processes, including cell survival and proliferation. Since IGF-1 is known to play a central role in the regulation of adult hippocampal neurogenesis [162], this finding may provide additional insight into the unique contribution of Rit signaling to immature neuron survival in the hippocampus, and have implications for the direction of the laboratories future research (Fig. 7.1).



**Figure 7.1 Physiological Functions of Rit Small GTPase.** Studies from this dissertation along with our previously published studies revealed that Rit may involve in multiple physiological processes especially in neurons. In response to oxidative stress, Rit controls a p38-MK2-HSP27 signaling cascade, which appears to act upstream of the mTORC2 to control Akt-dependent cell survival. The molecular mechanism of how Rit/p38 regulates mTORC2 activity, however, remains to be characterized. Meanwhile, Rit appears a key regulator of IGF-1 signaling, particularly ERK activation. This finding indicates Rit may play a significant role in IGF-1 mediated biological processes, including adult neurogenesis. Furthermore, our previous studies suggested Rit controls both ERK and p38 MAPK in response to NGF to mediate neurite outgrowth. However, the mechanism of how p38 regulates neurite outgrowth remained elusive. Data from this dissertation indicated Rit signaling can be coupled to mTORC2 kinase activity in a p38-dependent fashion. Therefore, Rit may regulate neurite outgrowth and axonal/dendritic morphology through mTORC2/PKC pathway.

### Evolutionary Conserved Pro-Survival Effect of Rit

Previous studies in cultured cell lines suggested that Rit was involved in multiple cellular activities. In NIH-3T3 cells, constitutively active Rit<sup>Q79L</sup> causes moderate tumorigenic transformation [98]. In PC6 cells, Rit plays a key role in NGF-induced neurite outgrowth in an ERK- and p38 MAPK-dependent manner [95]. Studies in primary neurons overexpressing constitutively active or dominant negative Rit mutants revealed that Rit activation promotes axonal elongation and at the same time inhibits dendritic elongation, while blockade of Rit signaling using the dominant-negative mutant had the opposite effect, promoting dendritic elongation [109]. These data implicate Rit as a key regulator of neuron differentiation and morphogenesis. In addition, Rit also appears to contribute to a survival signaling in cultured cell models including PC6 cells and HeLa cells [112]. However, the principle physiological role for Rit remained to be characterized. We decided to use a genetic approach to address this question, and generated a *Rit1* gene knockout mouse, with the expectation that this approach would provide a more accurate reflection of the physiological functions of Rit at both cellular level and whole animal level, than studies using RNAi approaches in transformed cell lines. We found that the Rit knockout mice were born at the expected Mendelian ratio, and grew to adulthood without showing any discernible abnormalities, suggesting that Rit does not control an essential aspect of cellular embryogenesis or development.

One of the exciting findings of this dissertation is that Rit mediates a p38 MAPK dependent pro-survival signaling in response to oxidative stress. Primary Rit<sup>-/-</sup> MEFs are specifically more vulnerable to H<sub>2</sub>O<sub>2</sub> exposure, but importantly not either ER stress or DNA damage, indicating that Rit is a key player in cellular oxidative stress resistance (Fig. 3.2). Overexpression of GFP-tagged active Rit<sup>Q79L</sup> restored cellular resistance of MEFs to H<sub>2</sub>O<sub>2</sub> exposure, supporting the protective effect (Fig.3.3). Since ROS is inevitable in aerobic organisms, and Rit is ubiquitously expressed, it is plausible to hypothesize that Rit functions to regulate a general cellular defense mechanism against oxidative stress, serving to protect a wide range of cells in the body against oxidative damage. A caveat to this notion is that Rit survival appears to rely upon p38 activation (Fig. 3.5), and the p38 MAPK cascade is known to promote both cell death, as well as cell survival. It will be important to examine the contribution of Rit to cell survival in

additional cell systems, especially neurons, cardiomyocytes, and granulocytes, in which p38 signaling has been shown to support survival [60, 265, 266]. Indeed, our studies have demonstrated a role for Rit in survival of at least one population of neurons. Loss of Rit results in primary hippocampal neurons, particularly immature hippocampal neurons, becoming hyper-sensitive to H<sub>2</sub>O<sub>2</sub> exposure (Fig. 5.5). More importantly, we have extended these *in vivo* studies, demonstrating increased vulnerability of Dcx<sup>+</sup> newborn immature granule neurons in the hippocampal DG following controlled cortical impact, a brain injury known to cause neuron loss due to excessive ROS in the brain (Fig. 5.8). Besides traumatic brain injury, both seizure and stroke are known to result in ROS accumulation, and elevated ROS levels have been reported in patients and in animal models of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease [267, 268]. Therefore, it will be important in future studies to examine the role of Rit in response to additional common brain injuries as well as neurodegenerative diseases. We predict that Rit loss may exacerbate the neuron loss seen under these pathophysiological conditions. As mentioned above, based on our current understanding of Rit-mediated pro-survival signaling, it is also worth expanding our future investigations beyond the central nervous system. Increased ROS levels, and consequent cell loss, are one of the signature features of cardiovascular-related disorders including atherosclerosis [258]. If the Rit-dependent protective function also proves critical for cardiovascular system under these pathological conditions, these data could provide a promising new avenue for the development of novel therapies for cardiovascular disease.

### **p38 MAPK and Rit-Mediated Pro-Survival Signaling**

We provide additional data supporting the importance of p38 MAPK, rather than ERK, signaling to Rit-dependent protection. This was quite surprising, since ERK1/2 is considered one of the major pro-survival MAPKs while p38 MAPK as well as JNK1/2 are well known as pro-apoptotic, and our studies have found a role for Rit in both p38 and ERK regulation [95, 193]. Recently, however, a growing body of evidence has shown that p38 MAPK may also function as a protective factor in some scenarios, indicating that the molecular mechanism of p38 activation may help to determine cell fate, and that whether p38 activation results in cell death or survival may be both cell

type- and stress-dependent. The opposing cellular actions of p38 MAPK may complicate ongoing therapeutic strategies based upon p38 MAPK inhibition. Indeed, p38 specific inhibitors have been shown to have cellular toxicity, particularly within the central nervous system [60]. Future studies elucidating the molecular mechanisms of Rit stress sensing and p38-Akt activation may provide new insight into p38-mediated cell survival and have implications for avoiding adverse clinical outcomes in the application of p38 inhibitors.

One of the recently identified p38-dependent pro-survival signaling pathways is the p38-MK2-HSP27-Akt signaling complex [127]. We have shown that Rit, but not Ras or Rap, specifically associates with this complex, making Rit the only small GTPase to date with the potential to directly control this scaffolded complex [112]. Studies in this dissertation have demonstrated that Rit-p38-MK2-HSP27-Akt signaling appears to be selectively activated in response to ROS signaling, but is not significantly engaged by growth factor signaling, including following EGF and bFGF stimulation (Figs. 3.4, 3.6 & 6.1). Despite this progress, several important questions remain to be addressed regarding Rit control of this signaling complex in response to ROS.

The first of these issues is how elevated ROS levels leads to Rit activation. Both Rit and p38 MAPK are known to be activated by a variety of extracellular stimuli including growth factors, cytokines as well as environmental stresses [57, 95, 107, 112, 183]. Among these stimuli, stress induced activation of small GTPases and MAPKs do not involve classic receptor tyrosine kinase or G-protein coupled receptor signaling, and to date, these activation cascades remain poorly understood. Elucidation of the mechanism of environmental stress-mediated Rit activation, especially identification of the responsible GEF(s) is an important goal of future studies. Rit<sup>-/-</sup> MEFs stably transfected with GFP-tagged dominant negative Rit (Rit<sup>S35N</sup>) could be a useful system to address this question, since Rit<sup>S35N</sup> is predominantly GDP-loaded and reports suggest that it should preferentially interact with RitGEFs. GFP pull-down followed by mass spectrometry might provide a strategy to identify RitGEFs. Alternatively, fluorescence resonance energy transfer (FRET) assays could be used to screen a collection of candidate GEFs. For this analysis we would need to construct two chimera fusion proteins: CFP-fused wild-type Rit and YFG-fused to a high affinity/specificity Rit-

binding domain from a known Rit effector (e.g. RGL3) [94]. FRET would provide a rapid *in vivo* read-out of Rit activation, and might allow both subcellular location of activation, and the kinetics of Rit stimulation to be addressed. In theory, overexpression of GEFs would induce Rit activation, while knock-down of specific GEFs with lentiviral shRNAs would disrupt Rit activation following stimuli. Therefore, if the proper system could be established, screening a library of GEFs should allow the identification of stimuli-specific RitGEFs.

A second pressing question is how Rit couples to p38 MAPK. It is well accepted that small Ras family GTPases regulate MAPKs, serving to link cellular stimuli to the activation of a three tier kinase cascade [53]. p38 MAPK is directly activated by the MAPKKs, MKK3/6 [57]. However, these kinases have been shown to be regulated by a large group of MAPKKKs, including MEKKs, ASK1, TAK1 and MLKs [57]. This reverse pyramidal hierarchy suggests that cells may utilize different MAPKKKs to specify upstream stimuli to control p38 activity. To facilitate signaling specificity, scaffolding proteins are known to play key roles in p38 signaling. One of the best examples of this regulation is the importance of OSM scaffolded Rac-MEKK3-MKK3 complex in osmotic stress-mediated p38 activation [62]. It is possible that Rit may also control p38 signaling through a scaffolded complex in response to oxidative stress. Indeed, the HSP27 scaffold is an attractive candidate, and studies directed at examining this possibility are underway in the laboratory.

It is also important to determine the downstream targets of the Rit-p38-MK2-HSP27-Akt signaling cascade required for cellular survival. Upon activation, Akt acts as a protein kinase to phosphorylate and either activate or inhibit a variety of downstream targets to promote survival [114]. Akt directly phosphorylates Bad and caspase-9 to block their pro-apoptotic activity [116, 117]. Indeed, the data in Chapter Three from this dissertation found that Rit-Akt signaling regulated Bad phosphorylation following H<sub>2</sub>O<sub>2</sub> exposure (Fig. 3.6D), suggesting that Rit inhibits Bad pro-apoptotic activity through the p38-MK2-HSP27-Akt signaling complex. Akt regulates mTOR kinase activity through the mTORC1 complex, which in turn initiates multiple pro-survival pathways [167]. In addition, Akt phosphorylates and regulates a number of transcription factors including FoxO, which is critical for oxidative stress-induced cell death [118]. Control of gene

expression is likely a major aspect of Rit-mediated survival signaling. Indeed, earlier microarray studies using *Ric*<sup>-/-</sup> *Drosophila* found that *Ric* loss leads to altered gene expression even in unstressed animals. More intriguingly, approximately 30% of these genes are known to have oxido-reductase, glutathione transferase and cytochrome P450 enzyme activity, indicating the pivotal role of Rit/*Ric* signaling in cellular ROS scavenger capacity regulation and therefore overall oxidative resistance. However, *Drosophila* may not accurately reflect the role of Rit in mammalian cells. Therefore, studies should be undertaken to examine gene transcription patterns in *Rit*<sup>-/-</sup> cells (MEFs and neurons) compared with wild-type controls at base line and after oxidative stress exposure. These data should provide insight into both the cellular mechanism for Rit-mediated survival, but also for the identification of Rit-regulated transcription factors.

Finally, we need to determine whether other p38 MAPK-dependent pro-survival pathways operate downstream of Rit. Indeed, recent unpublished data from our lab suggest that Rit controls a p38-MSK1/2-CREB signaling cascade. Loss of Rit in MEFs showed impaired MSK1/2 and CREB phosphorylation in response to H<sub>2</sub>O<sub>2</sub>, suggesting that the CREB transcription factor may play an important role downstream of Rit signaling. In addition, GSK3 $\beta$ , another well-known pro-apoptotic protein, has been reported to be phosphorylated and inactivated by p38 MAPK [224]. It will be interesting to ask whether Rit is involved in p38-GSK3 $\beta$  signaling following environmental stress exposure.

### **mTORC2 and Rit-Mediated Pro-Survival Signaling**

Although the data is still preliminary, studies in Chapter Four strongly suggest that Rit contributes to mTORC2-Akt signaling to promote cell survival. H<sub>2</sub>O<sub>2</sub> exposure and overexpression of constitutively active *Rit*<sup>Q79L</sup> stimulates mTORC2 kinase activity, resulting in phosphorylation of Akt at Ser<sup>473</sup> residue (Fig. 4.1). Moreover, loss of mTORC2 generated a similar sensitization of cells to oxidative stress as first seen in *Rit*<sup>-/-</sup> MEFs, suggesting a possible Rit-mTORC2-Akt pro-survival signaling pathway (Fig. 4.6). Data from this dissertation demonstrated that active Rit can directly interact with mTORC2 through Sin1 (Fig. 4.4). However, whether this interaction is required for mTORC2 kinase activation and subsequent mTORC2-mediated biology remains unclear.



Sin1 has a Raf-like Ras-binding domain, which has been reported to be responsible for Ras GTPases binding [237]. Studies of the Ras-binding domain (RBD) revealed that a conserved Arginine residue (Arg<sup>312</sup>) is required for Ras binding. Therefore, it will be important to test whether a Sin1 mutant (Sin1<sup>R312L</sup>) fails to interact with Rit, and if a loss of Sin1 binding results in the decoupling of Rit-dependent mTORC2 activation. Using this point mutation of Sin1, we will be able to examine whether Rit-Sin1 association is critical for mTORC2 kinase activity, and whether Sin1 association plays a central role in Rit-mediated protection against oxidative stress.

Sin1 also contains a C-terminal PH domain which serves to recruit mTORC2 to the cell membranes in a PI3K-dependent manner [237]. It is interesting to ask what roles the PH and RBD domains play in mTORC2 kinase subcellular localization and activation. Mutations of Sin1 (Sin1 $\Delta$ PH and Sin1<sup>R312L</sup>) could be used for this analysis. It is possible that PH domain-mediated mTORC2 translocation and activation is required for growth factor/receptor tyrosine kinases signaling, while the RBD domain plays a major role in mTORC2 membrane recruitment and activation following Rit activation in response to oxidative stress. Alternatively, they may play distinct roles in subcellular localization and the regulation of mTOR activity. Further studies are warranted to test these hypotheses.

Regulation of mTORC2 activity is complex, and recent studies have revealed that Ser<sup>2448</sup> phosphorylation of mTOR is correlated with increased mTORC1 complex activity, while phosphorylation at Ser<sup>2481</sup> is correlated with mTORC2-specific activity [240]. The kinases that direct these specific phosphorylation events, however, have not been defined. Similarly, multiple phosphorylation events on Rictor have also been reported to regulate mTORC2 kinase activity [269-271]. Future studies will need to identify the mechanism of Rit-dependent mTORC2 control. It has been reported that p38 interacts with the C-terminus of Sin1 [239]. This finding motivates the hypothesis that Rit may control mTORC2 kinase activity through p38 MAPK-mediated phosphorylation. Sin1 truncations ablating p38 binding could be used to test whether direct p38 association is required for H<sub>2</sub>O<sub>2</sub>/Rit-dependent mTORC2 activation. If true, it will be important to examine the phosphorylation profiles of mTORC2 following H<sub>2</sub>O<sub>2</sub> exposure, in the hope of identifying the p38-dependent phosphorylation events critical for mTORC2 activation.

The lack of mTORC2 specific inhibitors has made it difficult to study mTORC2-mediated signaling. Rapamycin specifically inhibits mTORC1 signaling. However, it acts by binding Raptor, leading to the disassembly of mTORC1 without altering mTOR kinase activity and therefore not altering mTORC2 complex activity [230]. Recently, the discovery of an efficient ATP-binding competitive inhibitor for mTOR makes pharmacological inhibitor studies feasible for mTORC2. By comparing rapamycin treatment alone, with co-treatment of rapamycin and an mTOR kinase inhibitor, it is possible to identify mTORC2 specific targets [272, 273]. Importantly, this combination inhibitor strategy may prove useful for studying the role of mTORC2 in Rit-mediated biology.

mTORC2 is a key regulator of AGC kinase family members including Akt, SGK and PKC within their conserved hydrophobic regulatory motif. All of these kinases are known to play crucial cellular roles including cell survival, energy metabolism, cytoskeleton dynamics and cell motility [226, 274]. Since Rit can stimulate mTORC2 activity, it will be interesting to investigate the possible roles of Rit in these biological processes. For instance, both Rit and PKC are known to regulate neurite outgrowth in neuronal cell lines as well as primary neurons [102, 109, 111, 242]. This suggests that Rit may regulate mTORC2-PKC pathway to control neurite outgrowth, which will be important future research direction.

### **Rit and Adult Neurogenesis**

Adult neurogenesis is a recently discovered physiological activity in the adult brain, which is responsible for the continuous production of new neurons in the central nervous system. Two major niches with high levels of adult neurogenesis have been identified in vertebrates, the SGZ in the hippocampal DG and SVZ of the lateral ventricles[137]. Adult neurogenesis in the SGZ is of particular interest since it appears to play a critical role in new memory formation [135]. In addition, adult neurogenesis appears to play a role in recovery/repair following brain injury-induced hippocampal neuron loss [181, 182]. The data included in this thesis indicates that Rit is a regulator of adult neurogenesis in the SGZ, particularly following traumatic brain injury, by regulating the survival of newborn hippocampal neurons.

Following controlled cortical impact, an experimental model of TBI, Rit loss selectively exacerbates the loss of Dcx<sup>+</sup> immature neuron within the DG, strongly suggesting that Rit signaling plays a protective role in this neuron population after injury (Fig. 5.8). This conclusion was supported by *in vitro* studies showing that Rit loss selectively sensitizes Dcx<sup>+</sup> immature neurons to H<sub>2</sub>O<sub>2</sub> (Fig. 5.5). TBI results in an acute and massive loss of immature neurons in the DG, compromising the very population of cells poised to compensate for trauma-induced neuronal loss. And recent work suggests that the loss of these cells following TBI exacerbates the resultant cognitive dysfunction [181]. Thus, these studies suggest that Rit signaling may facilitate brain recovery after the injury. However, more questions remain to be answered. In addition to newborn immature neurons, neural stem cells (NSCs) have also been shown to be vulnerable, including TBI [175]. Our *in vitro* culture studies indicate that Rit promotes the survival of immature neurons, but not nestin<sup>+</sup> NSCs, following oxidative stress (Fig. 5.5). Whether Rit contributes to neural stem cell survival following TBI *in vivo* remains to be examined. The study is technically challenging due to the lack of effective immunostaining protocols allowing for neural stem cell quantification. To address this question, it is likely that our Rit<sup>-/-</sup> mice will need to be crossed with Nestin-GFP transgenic mice, which express GFP in nestin<sup>+</sup> neural stem cells within hippocampal dentate gyrus [141].

The molecular mechanism(s) underlying Rit-mediated neuron survival following TBI remain to be addressed. The data from this dissertation shows that loss of Rit impairs both p38 and Akt phosphorylation after H<sub>2</sub>O<sub>2</sub> exposure, implicating p38-MK-HSP27-Akt signaling cascade in Rit-dependent survival signaling (Fig. 5.3). More studies are needed to determine, as discussed above, whether additional signaling pathways are involved. It would not be surprising if p38-MSK1/2-CREB signaling, or other cascades were also shown to contribute to neural survival. To confirm that Rit activation is alone sufficient to promote neuronal survival, it will be necessary to generate an inducible Rit<sup>Q79L</sup> expressing transgenic mouse line. With this model, it would be possible to test whether active Rit expressing neurons display increased resistance following TBI, and to confirm the importance of specific downstream effector pathways in Rit-mediated pro-survival signaling.

Based upon the studies described in this dissertation, Rit loss impairs IGF-1, but not BDNF, signaling in hippocampal neurons (Figs. 6.3 & 6.4), supporting a role for Rit in IGF-1-mediated signal transduction. IGF-1 is known to promote the proliferation and survival of multiple cell types, including important roles in regulation of the central nervous system [162, 253, 254, 256]. TBI transiently upregulates brain IGF-1 level [275], which can lead to a robust increase in the proliferation of neural stem cells [167]. This pathway appears to represent an endogenous neuroprotective or repair mechanism, and we hypothesize that Rit may play an important role in this cascade. To test this notion, a series of studies will be needed. First, we need to track the proliferation of neural stem cells shortly after TBI. By tracking the number of BrdU<sup>+</sup> cells co-labeled with specific neuronal markers (Nestin, Dcx and NeuN), we should be able to assess the contribution of Rit to the TBI-induced upregulation of adult neurogenesis. We expect that Rit loss will attenuate TBI-induced adult neurogenesis. If so, it will then be important to determine if Rit loss impairs adult neurogenesis induced by exogenous IGF-1 administration in naïve or brain-injured mice.

IGF-1 has also been shown in an array of *in vivo* studies to involve in exercise-induced adult neurogenesis and neuron survival [159, 163, 164]. Physical exercise induces synthesis and secretion of IGF-1 in the liver and boosts the level of circulating IGF-1, which enters central nervous system by crossing blood-brain barrier. As a result, physical exercise leads to increased IGF-1 in the brain, including hippocampus, which may be responsible for the survival of neurons as well as the enhancement of the proliferation and differentiation of neural stem cells in the hippocampal DG. Therefore, it will be interesting to ask whether Rit is involved in physiological stimuli such as exercise to modulate adult neurogenesis. Understanding the potential role of Rit in IGF-1 signaling may have great clinical significance. Decreased expression level of cerebral IGF-1R in the patients with Alzheimer's disease suggests that patients with neurodegenerative diseases may have obtained some level of IGF-1 resistance, which could hamper the effect of direct IGF-1 administration [276, 277]. Novel therapies for neurodegenerative diseases may be developed through selectively targeting Rit signaling, thus bypassing IGF-1R.

In addition, IGF-1 signaling has also been suggested to play important roles in some other disease processes including supporting cancer development [254]. Future studies will investigate whether Rit also contributes to IGF-1 signaling-dependent cancer cell proliferation.

### **Rit in Neuron Morphogenesis**

Our earlier studies have identified a critical role of Rit in neurite outgrowth in PC6 cells [95, 107], suggesting Rit is a key regulator of neuronal differentiation. In addition, ectopic overexpression of constitutively active and dominant negative Rit mutant in primary neuron cultures has revealed that the fine regulation of Rit activation in primary neurons may contribute to the control of neuron morphology [109]. Active Rit is able to induce axonal extension while inhibiting dendritic elongation, with the opposite effects resulting from dominant-negative Rit expression in primary cultured neurons. Taken together, these data indicate that Rit may play an important role in neuron morphogenesis. Therefore, it is important in future to examine in more detail the morphology of Rit null neurons. Golgi-Cox stain is a proven method for examining neuron morphology, including quantification of the dendrite number, branch complexity and length, as well as dendritic spine density and morphology [278]. Mechanistic studies indicate that both p38 and ERK MAP kinases are involved in Rit-mediated neurite outgrowth [95]. We have now shown that both Rit and p38 contribute to mTORC2 kinase activity regulation (See Chapter Four). Collectively, it is plausible to ask whether Rit-p38-mediated neurite outgrowth and neuron morphogenesis requires mTORC2 as a downstream target, since mTORC2 has been shown to regulate cytoskeleton rearrangement, regulating cell motility and morphology in multiple cell models from single cell organisms to vertebrates [133, 279-282]. Future studies could be directed at examining the role of Rit-mTORC2 signaling in primary neuron cultures as well as neuronal cell lines.

In conclusion, the data presented in this dissertation has identified a novel and unexpected physiological role for Rit in promoting cell survival in response to oxidative stress. Importantly Rit selectively promotes newborn immature neuron survival following

traumatic brain injury, which, to our knowledge, makes Rit the first small GTPase shown to play a critical role in the survival of a select population of developing neurons. This Rit-mediated pro-survival pathway appears to be dependent upon p38 MAPK-MK2-HSP27-Akt signaling complex in a fashion that depends upon mTORC2 activity. Future studies will need to investigate the upstream signals that controls stress-dependent Rit activation, the molecular mechanisms that couple Rit to p38 MAPK and its coupling to potentially distinct downstream signaling complexes, and the specific cellular targets that underlie Rit-mediated survival. In addition, this work has revealed a previously unrecognized but critical role for Rit in IGF-1 signaling, suggesting Rit a key regulator of IGF-1-mediated biology including adult neurogenesis. Future studies will be needed to investigate the contribution of Rit to adult neurogenesis, which may provide novel avenues for the development of new therapeutic strategies for the treatment of patients suffering from brain injuries and neurodegenerative diseases.

## Appendix

### List of Abbreviations

4E-BP1	Elongation initiation factor 4E-binding protein 1
AC	Adenylate cyclase
AF-6	ALL (acute lymphoblastic leukemia)-1 fused gene on chromosome 6
AGC family	Protein kinase A/G/C family
aPKC	Atypical protein kinase C
AraC	Cytosine arabinoside
Arf	ADP-ribosylation factor
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BMP7	Bone morphogenetic protein 7
BrdU	3'-bromodeoxyuridine
BSA	Bovine serum albumin
cAMP	cyclic AMP (adenosine monophosphate)
CCI	Controlled cortical impact
Cdc25	Cell division cycle 25
CMV	Cytomegalovirus
CREB	cAMP-response element binding protein
CTR	Control
DAG	Diacylglycerol
Dcx	Doublecortin
DIV	Days <i>in vitro</i>
DG	Dentate gyrus
DMEM	Dulbecco's modified Eagle's medium
DNA-PK	DNA protein kinase
DTT	Dithiothreitol
EGF	Epithelial growth factor
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated protein kinase
ES	Embryonic stem cell
ET	Etoposide
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FJC	Fluor Jade-C
FoxO	Forkhead O transcription factor
FRET	Fluorescence resonance energy transfer
GAP	GTPase-activating protein

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCV	Ganciclovir
GDI	Guanine nucleotide dissociation inhibitor
GDNF	Glial cell line-derived neurotrophic factor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HA	Hemagglutinin
HSP27	Heat shock protein 27
hsvTK	Thymidine kinase enzyme from herpes simplex virus
IFN- $\gamma$	Interferon- $\gamma$
IGF-1	Insulin-like growth factor
IGF-1R	IGF-1 receptor
I $\kappa$ -B	Inhibitory factor for NF- $\kappa$ B
IKK	I $\kappa$ -B kinase
IP	Immunoprecipitation
i.p.	Intraperitoneally
IP <sub>3</sub>	1,4,5-trisphosphate
IPTG	Isopropyl- $\beta$ -D-thiogalactopyranoside
IRS-1	Insulin receptor substrate-1
JIP	JNK-interacting protein
JNK	c-Jun N-terminal kinase
KSR	Kinase suppressor of Ras
MAP2	Microtubule associated protein 2
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MEK	MAP kinase kinase/ERK kinase
MK2	MAPK-activated protein kinase 2
MLK	Mixed lineage kinase
MST1/2	Mammalian Ste20-like kinase 1/2
mTOR	Mammalian target of rapamycin
mTORC1/2	Mammalian TOR complex 1/2
NeuN	Neuronal Nuclei
NF- $\kappa$ B	Nuclear factor $\kappa$ B



NGF	Nerve growth factor
NGS	Normal goat serum
NSC	Neural stem cell
NT-3	Neurotrophin-3
OSM	Osmosensing scaffold for MKK3
PACAP38	Pituitary adenylate cyclase-activating polypeptide-38
PAGE	polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PC6	Pheochromocytoma 6 cell line
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PH	Pleckstrin homolog domain
PI3K	Phosphatidylinositol-3 kinase
PKC	Protein kinase C
PLC	Phospholipase C
PSA-NCAM	Polysialylated neural cell adhesion molecule
Rab	Ras-related GTP-binding protein
Ran	Ras-related nuclear protein
Raptor	Regulatory associated protein of mTOR
Ras	Rat sarcoma
RASSF	Ras-association containing protein family
RBD	Ras-binding domain
Rho	Ras homolog gene family
Ric	Ras-related protein which interacts with calmodulin
Rictor	Rapamycin-insensitive companion of mTOR
Rin	Ras-related protein in neurons
RIN1	Ras interaction protein-1
Rit	Ras-related protein in tissues
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription-PCR
S6K1	Ribosomal protein S6 kinase 1
SAPK	Stress-activated protein kinase
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error mean
SGK	Serum/glucocorticoid regulated protein kinase
SGZ	Sub-granular zone

SH2	Src homology 2 domain
SH3	Src homology 3 domain
shRNA	small hairpin RNA
Sin1	SAPK-interacting protein 1
SOD	Superoxide dismutase
Sos1	Son of sevenless 1
SV40	Simian virus 40
SVZ	Sub-ventricular zone
TAK1	transforming growth factor $\beta$ -activated kinase 1
TBI	Traumatic brain injury
TM	Tunicamycin
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
TrkA	Neurotrophic tyrosine kinase receptor A
TrkB	Neurotrophic tyrosine kinase receptor B
UPS	Ubiquitin-proteasome system
VEGF	Vascular endothelial growth factor
WT	Wild-type

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## VITA

**Weikang Cai**

**Born: November 9, 1982**

**Birthplace: Changzhou, Jiangsu, P.R.China**

## EDUCATION

2005-Present	University of Kentucky College of Medicine Lexington, Kentucky
2001-2005	Nanjing University Nanjing, Jiangsu, P.R.China Bachelors of Science in Biochemistry

## ACADEMIC HONORS

2002 – 2004	Chinese People's Scholarship
2007	Academic Excellence Award
2007	Research Challenge Trust Fund I Fellowship
2008	Kentucky Opportunity Fellowship
2008	Thomas C. Vanaman Graduate Student Best Talk Award, 2008 Summer Research Conference, University of Kentucky, Department of Molecular and Cellular Biochemistry
2009	Max Steckler Fellowship
2010	Thomas C. Vanaman Graduate Student Best Talk Award, 2010 Summer Research Conference, University of Kentucky, Department of Molecular and Cellular Biochemistry

## PUBLICATIONS

### Peer-Reviewd Articles:

**Cai, W.**, Rudolph, J.L., Harrison, S.M.W., Jin, L., Frantz, A.L., Harrison, D.A. and Andres, D.A. 2011. An Evolutionarily Conserved Rit GTPase-p38 MAPK Signaling Pathway Mediates Oxidative Stress Resistance. *Mol. Biol. Cell* 22:17 3231-3241.

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